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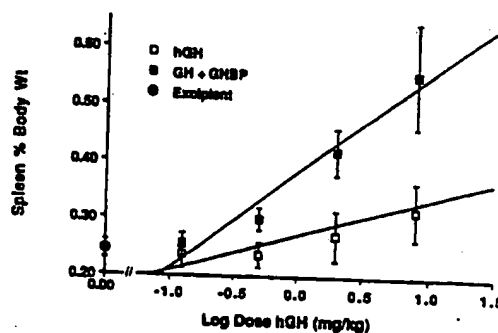
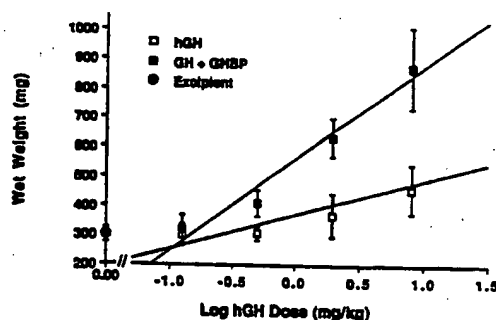
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(54) Title: METHOD OF STIMULATING IMMUNE RESPONSE USING GROWTH HORMONE

(57) Abstract

Methods are disclosed for stimulating a mammal's or avian's growth hormone responsive tissues, particularly immune response tissues in immune-compromised mammals, by administration of growth hormone or growth hormone plus insulin-like growth factor-I. Preferably, the growth hormone is pegylated growth hormone.



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METHOD OF STIMULATING IMMUNE RESPONSE USING GROWTH HORMONE

5

Background of the Invention

Field of the Invention

This invention relates to improved methods and compositions for the use of growth hormone (GH) which result in the continuous presence of serum levels of GH therapeutically effective in stimulating growth hormone responses in mammals or avians, including 1) increasing immune responses, such as antibody response to antigens in patients with suboptimal immune systems; and 2) novel therapeutic growth hormone administration methods and compositions that exhibit efficacy with intermittent administration.

15

Description of Related Art

Growth Hormone

A major biological effect of growth hormone (GH) is to promote growth in young mammals and maintenance of tissues in older mammals. The organ systems affected include the skeleton, connective tissue, muscles, and viscera such as liver, intestine, and kidneys. Growth hormones exert their effect through interaction with specific receptors on the target cell's membrane. hGH is a member of a family of homologous hormones that include placental lactogens, prolactins, and other genetic and species variants or growth hormone (Nicoll, C. S., et al. (1986) Endocrine Reviews 7, 169). hGH is unusual among these in that it exhibits broad species specificity and binds to either the cloned somatogenic (Leung, D. W., et al. [1987] Nature 330, 537) or prolactin receptor (Boutin, J. M., et al. [1988] Cell 53, 69). The cloned gene for hGH has been expressed in a secreted form in *Escherichia coli* (Chang, C. N., et al. [1987] Gene 55, 189) and its DNA and amino acid sequence has been reported (Goeddel, et al. [1979] Nature 281, 544; Gray, et al. [1985] Gene 39, 247).

Human growth hormone (hGH) participates in much of the regulation of normal human growth and development. This pituitary hormone exhibits a multitude of biological effects including linear growth (somatogenesis), lactation, activation of macrophages, insulin-like and diabetogenic effects among others (Chawla, R. K. (1983) Ann. Rev. Med. 34, 519; Edwards, C. K. et al. (1988) Science 239, 769; Thomer, M. O., et al. (1988) J. Clin. Invest. 81, 745). Growth hormone deficiency in children leads to dwarfism which has been successfully treated for more than a decade by exogenous administration of hGH.

Human growth hormone (hGH) is a single-chain polypeptide consisting of 191 amino acids (molecular weight 21,500). Disulfide bonds link positions 53 and 165 and positions 182 and 189. Niall, Nature, New Biology, 230: 90 (1971). hGH is a potent anabolic agent, especially due to retention of nitrogen, phosphorus, potassium, and calcium. Treatment of hypophysectomized rats with GH can restore at least a portion of the growth rate of the

rats. Moore *et al.*, Endocrinology, **122**: 2920-2926 (1988). Among its most striking effects in hypopituitary (GH-deficient) subjects is accelerated linear growth of bone-growth-plate-cartilage resulting in increased stature. Kaplan, Growth Disorders in Children and Adolescents (Springfield, IL: Charles C. Thomas, 1964).

- 5 hGH causes a variety of physiological and metabolic effects in various animal models including linear bone growth, lactation, activation of macrophages, insulin-like and diabetogenic effects and others (R. K. Chawla *et al.*, *Annu. Rev. Med.* **34**, 519 (1983); O. G. P. Isaksson *et al.*, *Annu. Rev. Physiol.* **47**, 483 (1985); C. K. Edwards *et al.*, *Science* **239**, 769 (1988); M. O. Thomer and M. L. Vance, *J. Clin. Invest.* **82**, 745 (1988); J. P. Hughes and H. G. Friesen, *Ann.*
- 10 *Rev. Physiol.* **47**, 469 (1985)). It has been reported that, especially in women after menopause, GH secretion declines with age. Millard *et al.*, Neurobiol. Aging, **11**: 229-235 (1990); Takahashi *et al.*, Neuroendocrinology, **46**: 137-142 (1987). See also Rudman *et al.*, *J. Clin. Invest.*, **67**: 1361-1369 (1981) and Blackman, Endocrinology and Aging, **16**: 981 (1987). Moreover, a report exists that some of the manifestations of aging, including decreased lean body mass,
- 15 expansion of adipose-tissue mass, and the thinning of the skin, can be reduced by GH treatment three times a week. See, *e.g.*, Rudman *et al.*, *N. Eng. J. Med.*, **323**: 1-6 (1990) and the accompanying article in the same journal issue by Dr. Vance (pp. 52-54). These biological effects derive from the interaction between hGH and specific cellular receptors. Two different human receptors have been cloned, the hGH liver receptor (D. W. Leung *et al.*, *Nature* **330**, 537
- 20 (1987)) and the human prolactin receptor (J. M. Boutin *et al.*, *Mol. Endocrinol.* **3**, 1455 (1989)). However, there are likely to be others including the human placental lactogen receptor (M. Freemark, M. Comer, G. Komer, and S. Handwerger, *Endocrinol.* **120**, 1865 (1987)). These homologous receptors contain a glycosylated extracellular hormone binding domain, a single transmembrane domain and a cytoplasmic domain which differs considerably in sequence and
- 25 size. One or more receptors are assumed to play a determining role in the physiological response to hGH.

Pegylation of Proteins

- hGH is given to children with growth deficiencies and proven to enhance growth.
- 30 However, the half-life of the injected hGH is so short that in order to have high efficiency as a pharmaceutical, hGH has to be injected at least three times a week and more commonly daily, which makes administering hGH a bigger task than some children can tolerate. The short half-life of hGH is believed to be due to its small molecular weight (22,000 dalton), and rapid renal clearance, which has been found to be proportional to the molecular weight of protein in
- 35 circulation. Pegylation, meaning conjugating polyethylene glycol (PEG) to protein, was found to be an excellent way to increase the molecular weight of protein. PEG is a non-immunogenic, linear, uncharged polymer with three water molecules per ethylene oxide unit therefore can alter the hydrodynamic properties of the conjugated molecules dramatically (Maxfield, *et al.*, *Polymer* **16**, 505-509 [1975]; Bailey, F. E., *et al.*, in *Nonionic Surfactants* [Schick, M. J., ed] pp.794-821,

1967)). Several enzymes for therapeutic usage were PEGylated to increase the *in vivo* half-life effectively (Abuchowski, A. et al, J. Biol. Chem. 252, 3582-3586, 1977; Abuchowski, A. et al, Cancer Biochem. Biophys. 7, 175-186, 1984). PEGylation of IL-2 (interleukin-2) was also reported to increase circulatory life as well as its potency (Katre, N.V. et al, Proc. Natl. Acad. Sci., 84, 1487-1491, 1987; Goodson, R. et al Bio/Technology, 8, 343-346, 1990). PEGylation of other molecules were reported to have reduced immunogenicity and toxicity (Abuchowski, A. et al, J. Biol. Chem., 252, 3578-3581, 1977).

Methods for covalently attaching polyethylene glycol to proteins was described in Davis et al. U.S. Pat. No. 4,179,337. Davis et al., disclose polypeptides such as enzymes and hormones coupled to polyethylene glycol or polypropylene glycol to obtain physiologically active non-immunogenic, water-soluble polypeptide compositions. One of the hormones named was growth hormone, however there were no examples showing any results with any growth hormone. The patented polypeptide compositions are prepared by a process involving the steps of reacting at least one terminal carbon atom bearing a hydroxy group from polyethylene glycol or polypropylene glycol with a coupling agent to provide an activated polymer having a reactive agent to provide an activated polymer having a reactive terminal group and reacting a physiologically active immunogenic polypeptide with the activated polymer by coupling the polypeptide to the reactive terminal group of the activated polymer. As the coupling agent, the patent discloses the use of some 13 polyfunctional coupling moieties including (a) cyanuric chloride or fluoride, (b) an acyl azide formed by reacting the polymer with chloroacetic acid, then with diazomethane to obtain the methyl ester of the carbomethoxy ether followed by treatment with hydrazine which gives the corresponding hydrazide which is then treated with nitrous acid to give the acylazide, (c) a dihalosuccinic anhydride, (d) the p-diazobenzyl group, (e) the 3-(p-diazophenyl)-hydroxy propyloxy group, (f) the 1-glycidyloxy-4(2-hydroxy-3-propyl) butane group, (g) carboxyamino or thiocarbonylamino benzyl linkage, (h) the 2-(hydroxy-3-carboxy) propyl linkage, (i) an ω -amino derivative produced from an alkylated PEG such as methoxy polyethylene glycol, subsequently reacted with a carboxy group of a polypeptide, and (j) coupling of an ω -amino derivative of an alkylated PEG with maleic anhydride and subsequent reaction of the N-PEG-maleimide with the desired polypeptide.

The Enzon, Inc. catalog, "Enzymes For Biomedical Use" dated May, 1983 disclosed the commercial availability of certain polyethylene glycol modified enzymes (referred to as "PEGzymes") having improved storage stability, resistance to proteolytic digestion, extended blood circulating times, and reduced immunogenicity. Also disclosed are two activated polyethylene glycol products for protein modification including monomethoxypolyethylene glycol succinimidyl succinate. A footnote states that, "For some enzymes such as asparaginases this coupling agent yields PEGzymes with higher specific activities."

Stabilization and modification of enzymes and other proteins by covalent attachment to carbohydrates and polyethyleneglycol has been reported. Marshall and Rabinowitz, Arch.

biochem. biophys., 167, 77 (1975) and J. Biol. Chem., 251, 1081 (1976), noting earlier reports that glycoproteins (mostly enzymes) often show unusual stability characteristics compared with carbohydrate-free proteins, the former being less sensitive to heat and other denaturing conditions and more resistant to proteolysis, disclose the preparation of soluble enzyme-carbohydrate conjugates by coupling (by means of covalent attachment) trypsin, alpha-amylase and beta-amylase to cyanogen bromide activated dextran. The resulting covalent conjugates displayed marked resistance to heat inactivation and denaturation, increased half-lives, and reduction in loss of activity under conditions favoring autolysis.

Therapeutic formulations containing hormones in combination with antibodies specific for the hormone were reported to potentiate the activity of the hormone provided that the epitope specificity of the antibody is chosen appropriately. This potentiation or mimicry of hormones, such as growth hormone, is disclosed in Astor et al., EP 137234B, 10 October 1990.

Abuchowski et al., J. Biol. Chem., 252 (11), 3578 and 3582 (1977), disclose the modification of proteins, specifically, bovine serum albumin and bovine liver catalase, by the covalent attachment thereto of nonimmunogenic methoxyethylene glycols of 1900 Daltons (PEG-1900, Union Carbide Corp.) and 500 Daltons (PEG-5000), Union Carbide Corp.) using cyanuric chloride (2,4,6-trichloro-s-triazine) as the coupling agent. The modified bovine serum albumin exhibited a blood circulating life in rabbits similar to native bovine serum albumin except that it was not removed from circulation by the eventual development of antibodies. Also, the modified bovine serum albumin exhibited substantial changes in properties, such as solubility, electrophoretic mobility in acrylamide gel, ion exchange chromatography, and sedimentation, as compared with the unmodified protein. Rabbits were immunized by the intravenous or by the intramuscular administration of PEG-1900-catalase. The intravenous administered antigen did not yield detectable antibodies against PEG-1900-catalase or native catalase whereas the antiserum from intramuscular administered antigen contained antibodies to PEG-1900-catalase and native catalase. PEG-5000-catalase did not react with either antiserum.

PEG-1900-catalase and PEG-5000-catalase retained 93% and 95%, respectively, of their enzymatic activity and PEG-5000-catalase resisted digestion by trypsin, chymotrypsin and a protease from Streptomyces griseus. PEG-1500-catalase and PEG-5000-catalase exhibited enhanced circulating lives in the blood of acatalasemic mice during repetitive intravenous injection and no evidence was seen of an immune response to injections of the modified enzymes.

35

Insulin-like Growth Factor I

Insulin-like growth factor I (IGF-I) is a polypeptide naturally occurring in human body fluids, for example, blood and human cerebral spinal fluid. Most tissues, and especially the liver, produce IGF-I together with specific IGF-binding proteins. IGF-I production is under the

dominant stimulatory influence of growth hormone (GH), and some of the IGF-I binding proteins are also increased by GH. See Tanner *et al.*, Acta Endocrinol., **84**: 681-696 (1977); Uthne *et al.*, J. Clin. Endocrinol. Metab., **39**: 548-554 (1974)). IGF-I has been isolated from human serum and produced recombinantly. See, e.g., EP 123,228 and 128,733.

5 The levels of IGF-I are reported to be reduced by half in 20-month old rats compared to 6-month old rats (Takahashi and Meiters, Proc. Soc. Exp. Biol. Med., **186**: 229-233 (1987)). See also Florini and Roberts, J. Gerontol., **35**: 23-30 (1980); Florini *et al.*, Mech. Ageing Dev., **15**: 165-176 (1981); Chatelain *et al.*, Pediatr., **44**: 303-308 (1989); Florini *et al.*, J. Gerontol., **40**: 2-7 (1985); Hall and Sara, Clinics in Endocrin. and Metab., **13**: 91 (1984); Baxter,
10 Advances in Clinical Chemistry, **25**: 49 (1986); Clemmons and Underwood, Clinics in Endocrin. and Metab., **15**: 629 (1986); Hintz, Advances in Pediatrics, **28**: 293 (Year Book Medical Publishers, Inc., 1981); Johanson and Blizzard, The Johns Hopkins Medical Journal, **149**: 115-117 (1981), the latter five references describing low IGF-I levels in aged men. The Hintz, Clemmons and Underwood, and Baxter references are general reviews on IGF-I.

15 Furthermore, it was found that among human diploid fibroblasts capable of cycling in aging cultures *in vitro*, there were few changes in the regulation of the growth fraction by platelet-derived growth factor (PDGF) and epidermal growth factor (EGF), but a greatly increased dependence on IGF-I for regulation of the rate of entry into S phase (Chen and Rabinovitch, J. Cell. Physiol., **144**: 18-25 (1990)). The authors conclude that the slower growth
20 of the dividing population of cells in aging cultures may be related to a requirement for IGF-I at levels that are greatly above those usually supplied. This may be due to overproduction of the IGF-I binding protein, IGFBP-3, and, therefore, a reduction in IGF-I availability to its receptor. Goldstein *et al.*, "Cellular and Molecular Applications to Biology of Aging", AFCR Meeting abstract, Seattle, May 4-5, 1991.

25 Various biological activities of IGF-I in other than aged mammals have been identified. For example, IGF-I is reported to lower blood glucose levels in humans (Guler *et al.*, N. Engl. J. Med., **317**: 137-140 (1987)). Additionally, IGF-I promotes growth in several metabolic conditions characterized by low IGF-I levels, such as hypophysectomized rats [Skottner *et al.*, J. Endocr., **112**: 123-132 (1987)], diabetic rats [Scheiwiller *et al.*, Nature, **323**: 169-171 (1986)],
30 and dwarf rats [Skottner *et al.*, Endocrinology, **124**: 2519-2526 (1989)]. The kidney weight of hypophysectomized rats increases substantially upon prolonged infusions of IGF-I subcutaneously (Guler *et al.*, Proceedings of the 1st European Congress of Endocrinology, **103**: abstract 12-390 (Copenhagen, 1987)). The kidneys of Snell dwarf mice and dwarf rats behaved similarly (van Buul-Offers *et al.*, Pediatr. Res., **20**: 825-827 (1986)); Skottner *et al.*,
35 Endocrinology, *supra*. An additional use for IGF-I is to improve glomerular filtration and renal plasma flow (Guler *et al.*, Proc. Natl. Acad. Sci. USA, **86**: 2868-2872 (1989)). The anabolic effect of IGF-I in rapidly growing neonatal rats was demonstrated *in vivo* (Philipps *et al.*, Pediatric Res., **23**: 298 (1988)). In underfed, stressed, ill, or diseased animals, IGF-I levels are well known to be depressed.

GH and IGF-I

GH and IGF-I have been linked with immunoregulatory properties. The immune response results from interaction of antigens (foreign or non-self moieties) with host cells (lymphocytes) bearing specific receptors on the surface membrane for these antigens. Lymphocytes are grouped into two major classes, T-cells and B-cells.

T-cells originate from the thymus where they mature and differentiate from bone-marrow-derived cells. The mature T-cells leave the thymus gland to continuously circulate from blood to lymph nodes and spleen and back to blood. T-cells are further subdivided into three major subsets: T-helper cells, T-suppressor cells, and T-cytolytic cells. T-helper cells "help" other cells: B-cells to secrete antibody, cytotoxic cells to become functional, and macrophages to become activated. This population of T-cells bears the CD₄ surface marker that is used to identify this subset in tissue and blood.

T-cytolytic cells are responsible for killing target cells such as virally infected cells, tumor cells, and allografts. Suppressor T-cells act to limit and terminate the immune response. The cytolytic and suppressor T-cell populations are identified by the CD₈ surface marker.

The B-cells, or antibody-forming cells, also derive from immature precursors found in the bone marrow. When mature, the B-cells migrate to all lymphoid organs except the thymus. B-cells interact with antigens by way of antibody molecules bound to their plasma membranes that act as receptor proteins. This surface immunoglobulin is used as a marker to identify B-cells in tissue and blood. Following interaction with antigen and T-helper cells, the B-cells differentiate into antibody-forming cells called plasma cells. These plasma cells secrete antibody into the extracellular matrix. The antibody diffuses into capillaries and circulates via normal blood flow. Thus, the serum immunoglobulin level reflects the cellular dynamics of the immune response.

In many states, children are required to be immunized routinely against such diseases as diphtheria, pertussis, and typhoid (DPT), as well as measles, tetanus, mumps, polio, and rubella, by administering vaccines. The B-cell reaction to vaccine is the production of appropriate immunoglobulins, which are intended to confer immunity against the disease. Generally, a particular B-cell will be differentiated to produce one particular type of antibody, and such production is caused by the presence in the body of one particular type of antigen. Hence, when an animal or person has been exposed to a number of different antigens, the animal or human will have a number of different B-cells that can produce its particular immunoglobulins when the appropriate antigen is present.

In some situations, the immune response to antigen is insufficient to confer immunity. That is, a quantity of immunoglobulins is generated (or a number of B-cells are potentiated) that is insufficient to confer effective immunity.

It has been known since 1967 that a connection exists between the anterior pituitary and the immune system, and specifically with GH. Two groups of investigators concluded from their studies that GH controls the growth of lymphoid tissue (Pierpaoli and Sorkin, Nature, 215: 834 (1967); Baroni, Experientia, 23: 282 (1967)). Subsequently, immunologic
5 function was restored in the pituitary dwarf mouse by a combination of bovine somatotrophic hormone and thyroxine (Baroni *et al.*, Immunol., 17: 303-314 (1969)).

In a sex-linked dwarf chicken strain, bovine GH treatment resulted in enhanced antibody responses and bursal growth while thyroxine treatment stimulated thymus growth (Marsh *et al.*, Proc. Soc. Exp. Biol. Med., 175: 351-360 (1984)). However, neither treatment
10 altered immune function in the autosomal dwarf chicken. Bovine GH therapy alone partially restored immunologic function in immunodeficient Weimaraner dogs (Roth *et al.*, Ann. J. Vet. Res., 45: 1151-1155 (1984)).

Mice with hereditary GH deficiency develop an impairment of the immune system associated with thymic atrophy, immunodeficiency, and wasting, resulting in a shortened life expectancy (Frabris *et al.*, Clin. Exp. Immunol., 9: 209-225 (1971)). It has been shown that an
15 age-associated decline in the plasma concentration of thymulin (a thymic hormone) occurs and that plasma thymulin concentration increases in bGH-treated middle-aged and old dogs (Goff *et al.*, Clin. Exp. Immunol., 68: 580-587 (1987)). The authors suggest that exogenous GH may be useful for restoring some immune functions in aged individuals. Further, administration of
20 hGH to C₅₇/B1/6J mice was found to reverse the inhibitory effect of prednisolone on thymus and spleen cellularity and on natural killer activity; administration of hGH without prednisolone had no effect, although at higher doses it induced a decrease of thymic parameters and natural killer activity with no effect on spleen cellularity, and relative weights (Franco *et al.*, Acta Endocrinologica, 123: 339-344 (1990)).

It has also been shown that GH induces T-cell proliferation in the thymus (Murphy *et al.*, FASEB Meeting Abstract, Atlanta, April 1991; Durum *et al.*, FASEB Meeting Abstract, Atlanta, April 1991). For recent reviews on the immune effects of GH, see Kelley, "Growth Hormone in Immunobiology," in Psychoneuroimmunology II, 2nd Ed., B. Ader *et al.*, eds., Acad. Press 1990, and Ammann, "Growth Hormone and Immunity," in Human Growth Hormone--
30 Progress and Challenges, L. Underwood, ed., Marcel Dekker, Inc., New York, (1988), pp. 243-253; and Weigent and Blalock, Prog. NeuroEndocrinImmunology, 3: 231-241 (1990). It has been reported that the activity of all major immune cell types, including T-cells, B-cells, natural killer (NK) cells and macrophages, can all be altered by GH (Kelly, Biochem. Pharmacol., 38: 705 (1989)).

One report states that locally generated IGF-I mediates GH action on T-lymphocytes through the type I IGF receptor (Geffner *et al.*, J. Clin. Endocrin. and Metab., 71: 464 (1990)). Also, Franco *et al.*, on p. 343, speculate that some of the effects of hGH on the immune system occur via IGF-I. Timsit *et al.* at the Endocrine Society 73rd Annual meeting, June 19-22, 1991, Abstract 1296 reports hGH and IGF-I stimulate thymic hormone production.

There have been data published documenting the ability of cells of the immune system to produce IGF-I-like molecules. These include activated alveolar macrophages [Rom *et al.*, J. Clin. Invest., **82**: 1685 (1988)], human B-lymphocytes transformed with Epstein-Barr virus [Merimee *et al.*, J. Clin. Endocrin. Metab., **69**: 978 (1989)], spleen and thymus tissues through
5 detection of mRNA for IGF-I [Murphy *et al.*, Endocrinology, **120**: 1279 (1987)], and normal T-cells [Geffner *et al.*, *supra*].

Data have also been presented suggesting that IGF-I produced locally in tissues such as the thymus or inflammatory sites might affect the growth and function of IGF-I-receptor-bearing T- lymphocytes (Tapson *et al.*, J. Clin. Invest., **82**: 950-957 (1988)). A statistically
10 significant increase in thymus and spleen weight of hypophysectomized rats infused for 18 days with IGF-I was observed as compared to control or treatment with GH (Froesch *et al.*, in Growth Hormone Basic and Clinical Aspects, eds. O. Isaksson *et al.*, p. 321-326 (1987)). Also reported was an increased thymic tissue in young GH-deficient rats treated with IGF-I [Guler
et al., Proc. Natl. Acad. Sci. USA, **85**: 4889-4893 (1988)] and an increase in the spleen of
15 dwarf rats [Skottner *et al.*, Endocrinology, *supra*]. Others have shown repopulation of the atrophied thymus in diabetic rats using either IGF-I or insulin; however, when the rats were immunized with bovine serum albumin (BSA) and boosted, there was no effect of insulin or IGF-I on the antibody response despite large effects on thymic and splenic size (Binz *et al.*,
Proc. Natl. Acad. Sci. (USA), **87**: 3690-3694 (1990)). IGF-I was reported to stimulate
20 lymphocyte proliferation (Johnson *et al.*, Endocrine Society 73rd Annual Meeting, June 19-22, 1991, Abstract 1073), 87.

Furthermore, IGF-I was found to repopulate the bone marrow cavity with hematopoietic cells [Froesch *et al.*, *supra*], stimulate erythropoiesis in hypophysectomized rats [Kurtz *et al.*, Proc. Natl. Acad. Sci. (USA), **85**: 7825-7829 (1988)], and enhance the maturation
25 of morphologically recognizable granulocytic and erythroid progenitors in suspension cultures of marrow cells (Merchav *et al.*, J. Clin. Invest., **81**: 791 (1988)).

At nanomolar concentrations, IGF-I is a growth-promoting factor for lymphocytes (Schimpff *et al.*, Acta Endocrinol., **102**: 21-25 (1983)). B-cells, but not T-cells, have recently been shown to possess receptors for IGF-I (Stuart *et al.*, J. Clinical Endo. and Met., **72**: 1117-
30 1122 (1991)). Also, IGF-I, as a chemotactic for resting and activated T-cells, stimulates an increase in thymidine incorporation into resting and activated T-cells. Normal T-cell lines show augmentation of basal colony formation in response to IGF-I (Geffner *et al.*, *supra*). It is also stated on p. 955 of Tapson *et al.*, J. Clin. Invest., **82**: 950-957 (1988) that IGF-I produced locally in tissues such as the thymus or inflammatory sites might affect the growth and function of
35 IGF-I receptor-bearing T lymphocytes. However, IGF-I is reported to suppress in a dose-dependent manner IL-2-induced proliferative responses and *in vitro* antibody responses of splenocytes (Hunt and Eardley, J. Immunol., **136**: 3994-3999 (1986)).

There is a need in the art to supply a reagent that will stimulate the immune system of a mammal or avian, whether the immune response is cell-mediated or antibody-mediated.

There is a particular need for a reagent that will boost the antibody response of patients with compromised immune systems to antigens to which they are exposed. In view of the controversy in the art surrounding IGF-I, it is unclear what its effects would be in increasing immune function, as opposed to merely increasing size of organs involved in immune function
5 such as the thymus and spleen, or in increasing the activity of T- or B-cells *in vitro* or *in vivo*.

It is therefore an object of the present invention to stimulate the immune response of a mammal or avian.

It is a particular object to increase production of immunoglobulins by increasing the number of immunoglobulin-producing cells and/or by increasing the amount of immunoglobulin
10 produced by the individual immunoglobulin-producing cells in response to the predetermined immunogen.

It is a more particular object to increase antibody responses in patients with severely hampered immune systems, such as patients who receive bone marrow transplants or in AIDS patients.

15 It is an object of the present invention to develop a method of preferentially stimulating the growth or development of the immune system through the administration of GH containing compositions.

It is an object of the present invention to devise methods of therapeutic use of GH containing compositions wherein the composition may be administered intermittently, such as
20 every three or more days. Other objects, features and characteristics of the present invention will become more apparent upon consideration of the following description and the appended claims. These and other objects will be apparent to those of ordinary skill in the art.

Summary of the Invention

25 Described is a method for the use of growth hormone comprising therapeutic methods which result in the continuous presence of a therapeutically effective amount of GH. In one application of this method, continuous GH presence can be achieved by the use of a catheter, insulin pump or implanted diffusion device which slowly administers a dose of GH which results in the stimulation of the immune system leading to an improved immune response. In a second
30 application of the method of the present invention, continuous presence of GH is achieved by coupling of GH to other macromolecules that results in an improved half-life of GH thereby facilitating the continuous presence of a therapeutically effective amount of GH. Among the preferred macromolecules that result in continuous presence of GH are 1) growth hormone binding protein; 2) covalently attached polymers such as polyethylene glycol, polypropylene
35 glycol or carbohydrates; and 3) other macromolecules such as proteins, lipids, or glycolipids that reduce clearance and are not immunogenic.

The continuous presence of GH when combined with a macromolecule causing an increased serum half-life results in a therapeutic composition which may be intermittently administered. Such intermittent administration may be once every three days or more, more

preferably every six days or more. Disclosed are novel modified forms of human growth hormone (hGH) having specific amino acid residues modified by covalently attaching polyethyleneglycol (PEG). Also described is the use of PEG-GH to achieve optimum growth with intermittent administration. Further disclosed is the therapeutic use of PEG-GH or GH, in combination with IGF-I, in stimulating the development of the immune system. Also disclosed are novel therapeutic hGH administration methods and compositions that exhibit efficacy with intermittent administration. Accordingly, the present invention provides a method for stimulating a mammal's or avian's immune system comprising administering to the mammal or avian an immune-stimulating effective amount of a GH containing composition. GH may be used in combination with IGF-I in a method of immune stimulation.

In a more particular aspect, the invention provides a method for increasing a mammal's or avian's antibody response to an immunogen comprising administering to the mammal or avian the immunogen and an effective amount of GH and IGF-I. This use of GH, PEG-GH, with or without IGF-I, may be considered as an endocrine or hormonal adjuvant for the immunization process. Preferably, this administration is concurrent and is followed by boosts of immunogen at shortened intervals relative to immunization methods when no GH and IGF-I are given. Therefore, the invention provides co-administration of effective amounts of IGF-I and GH for stimulating the immune system. This hormonal adjuvant, that is the use of GH or of GH plus IGF-I as an adjuvant to promote the immune response, is applicable to any antigenic substance. Most preferably the antigen is derived from microorganisms, viruses and tumors.

In still another aspect, a method is provided of increasing the amount of immunoglobulin produced by B-cells of a human or other mammalian subject in response to an immunogen, where said subject suffers from a condition in which insufficient immunoglobulin production occurs, comprising administering to the subject an effective amount of GH and IGF-I, the amount being effective to increase the production of immunoglobulin.

In a still further aspect, the invention provides a method of increasing the T-cell responsiveness in a human or other mammalian subject in response to an immunogen, where said subject suffers from a condition in which insufficient T-helper or T-cytolytic activity occurs, comprising administering to the subject an effective amount of GH and IGF-I, the amount being effective to increase the T-helper or T-cytolytic activity.

Studies with GH have shown that bone growth may be stimulated by regular bolus doses of GH, between the bolus doses the GH serum concentration falls to very low levels. Such bolus daily doses of GH are not effective at achieving a continuous GH presence sufficient to stimulate the immune system. The present invention discovered that the continuous presence of results in the stimulation of the immune system and that such continuous presence can be achieved by the use of a GH formulation.

The intermittent administration of GH is not a therapeutically effective use of GH to stimulate the immune system. Stimulation of the immune system requires the continuous

presence of GH. Intermittent administration of GH can result in the continuous presence of GH when the GH is complexed with itself or with another macromolecule such that the GH is not cleared from the plasma. Intermittent GH use is defined as administration every 3 or more days, preferably every 6 or more days. This unexpected use of GH in combination with a macromolecule such as PEG or covalent attachment to other large polymers results in a therapeutic composition that may be administered at intermittent intervals yet results in the continuous presence of GH in the plasma. Continuous presence GH results in the unexpected stimulation of the immune system. A second unexpected result of using GH complexed with a macromolecule is the stimulation of the growth of other tissues such as bone and muscle when compared to GH not complexed to a macromolecule and intermittently administered every 6 or more days. The administration of GH complexed to a macromolecule, such as PEG, thus surprisingly results in the stimulation of bone and other tissues even when GH administration is intermittent.

15 **Stimulation of Immune System**

Previously growth hormone secreted by administered GH secreting tumor cells has been shown to stimulate growth (Kelley, *Biochem Pharmacol.*, 38 705, 1989). Growth hormone and growth hormone receptor have been shown to be present within the cells of the immune system. However there has been no indication that GH administration would result in a therapeutically useful stimulation of the immune system of mammals or birds. The administration of GH alone, or GH in combination with IGF-I stimulates the immune system, most preferably when GH is continuously present in the blood plasma and body tissue fluids. Surprisingly, the continuous presence of GH results in the stimulation of immune responsive tissues, such as the thymus and spleen.

25 A preferred form of GH is GH in combination with a macromolecule that increases the plasma half-life of the GH. Such macromolecules include polyethylene glycol, polypropylene glycol, carbohyrates, lipids, and proteins. Among the preferred proteins are the GH binding proteins, antibodies and albumin. The most preferred form of GH is pegylated GH. Preferred formulations for administration to induce the continuous effective plasma concentrations of GH include GH with one or more pegylated lysine or methionine residues.

30 While recent studies in whole animals have shown that IGF-I can cause increased spleen and thymus weights in GH-deficient animals, these studies have not progressed beyond describing a gross change in thymus and spleen size or in cell number. Other manipulations of the size of the spleen and thymus have been shown not to be associated with an effect on function (Jardieu and Fraker, *J. Immunol.*, 124: 2650-2655 (1980)). Furthermore, the Binz *et al.* article cited above utilized a diabetic rat model where insulin and IGF-I would affect diabetes and therefore aid all tissues in the body, and IGF-I and insulin were found to have no functional effect on antibody titer.

In view of this art, the present invention represents an unexpected finding that not only are the spleen and thymus weights increased upon administration of GH containing compositions, but also the function of the thymus, spleen, or lymph nodes, as indicated by increased size and cell numbers in response to IGF-I. The increase in cell number and responsiveness translates to an increased production of antibody by these cells in response to an antigen. This method would be useful in treating patients having compromised immune systems such as AIDS patients, in whom increased antibody response to antigens would ward off, or decrease the severity of, infectious diseases and in whom vaccines could be made more effective.

Brief Description of the Drawings

Figure 1: figure 1A is a plot of the spleen weight gain in rats following treatment with various doses of excipient, GH or GH plus GHBP for 7 days; figure 1B is the same spleen data plotted as a percent of whole body weight.

Figure 2 is a graph of thymus weight in hypox rats following treatment with various doses of hGH, PEG-1 hGH or PEG-2 hGH.

Figure 3 is a graph of whole body weight gain after 24 days in hypox rats receiving treatment with excipient, PEG-5 daily, every 3 days or every 6 days; or hGH daily, every 3 days or every 6 days.

Figure 4 is a graph of the thymus weight following the same treatment as in figure 3.

Figure 5 is a time plot for 16 days of whole body weight gain following treatment with excipient, hGH every 6 days, hGH daily, PEG-hGH daily or PEG-hGH every six days. All groups received 0.1mg/kg/day of hGH.

Figure 6 is a graph of body weight gain in aged rats treated with excipient, IGF-I, hGH, or IGF-I plus hGH.

Figures 7A, 7B, and 7C provide graphs on the splenocyte number, splenic T-cell population number, and splenic B-cell number, respectively, after 7-day IGF-I treatment or excipient treatment.

Figures 8A, 8B, and 8C provide graphs on the splenocyte number, splenic T-cell population number, and splenic B-cell number, respectively, after 14-day IGF-I, GH or excipient treatment.

Figure 9 represents a graph of the number of thymocytes after 14-day IGF-I treatment, hGH treatment, IGF-I control treatment, and hGH control treatment.

Figure 10 represents a graph of the mitogenic responses 14 days after initial excipient or IGF-I or hGH treatment of mice using the mitogens LPS (Fig. 10A), Con A (Fig. 10B), or PWM (Fig. 10C).

Figures 11A, 11B, and 11C provide graphs on the splenocyte number, splenic T-cell population number, and splenic B-cell number, respectively, after 14-day treatment with excipient, IGF-I, hGH, and IGF-I plus hGH.

Figure 12 represents a graph of the number of thymocytes after 14-day IGF-I treatment, hGH treatment, and IGF-I plus hGH treatment.

Figures 13A, 13B, and 13C represent graphs of splenic lymphocyte number, splenic T-cell subpopulation number, and splenic B-cell number, respectively, 7 days after the end of excipient, IGF-I, hGH, and IGF-I plus hGH treatment.

Figure 14 represents a graph of the number of thymocytes 7 days after the end of excipient, IGF-I, hGH, and IGF-I plus hGH treatment.

Figure 15 represents a graph of the mitogenic responses 7 days after the end of excipient, IGF-I, hGH, or IGF-I plus hGH treatment of mice using the mitogens LPS (Fig. 15A), Con A (Fig. 15B), or PWM (Fig. 15C).

Figures 16A and 16B represent graphs of the lymph node cell number and lymph node T-cell populations, respectively, 7 days after the end of excipient, IGF-I, hGH, and IGF-I plus hGH treatment.

Figures 17A, 17B, and 17C provide graphs on the splenic lymphocyte number, splenic T-cell population number, and splenic B-cell number, respectively, 21 days after the end of excipient, IGF-I, hGH, and IGF-I plus hGH treatment.

Figure 18 represents a graph of the number of thymocytes 21 days after the end of excipient, IGF-I, hGH, and IGF-I plus hGH treatment.

Figure 19 represents a graph of the mitogenic responses 21 days after the end of excipient, IGF-I, hGH, or IGF-I plus hGH treatment of mice using the mitogens LPS (Fig. 19A), Con A (Fig. 19B), or PWM (Fig. 19C).

Figure 20 shows the concentration of anti-dinitrophenyl-ovalbumin IgG (Fig. 20A) and total IgG (Fig. 20B) in $\mu\text{g/ml}$ in the serum of mice as a function of the number of weeks since the first immunization with dinitrophenyl-ovalbumin conjugate (Day 0, designated AG), wherein at week 3 (Day 20) the mice were boosted with conjugate and given excipient or IGF-I.

Figure 21 shows the weight gain changes for mice with and without transplanted bone marrow and treated with excipient or 40 μg or 120 μg of IGF-I.

Figures 22A, 22B, and 22C show graphs of peripheral blood lymphocyte B-cells, T-cell subpopulations, and H/S ratio, respectively, 14 days after irradiation of mice with transplanted bone marrow and treated with excipient, 40 μg IGF-I, or 120 μg IGF-I.

Figures 23A, 23B, and 23C show graphs of splenic lymphocyte number, splenic T-cell subpopulations and splenic B-cell number, respectively, 14 days after irradiation of mice with transplanted bone marrow and treated with excipient, 40 μg IGF-I, or 120 μg IGF-I.

Figure 24 represents a graph of the mitogenic responses 14 days after irradiation of mice with transplanted bone marrow and treated with excipient, 40 μg IGF-I, or 120 μg IGF-I using the mitogens LPS (Fig. 24A), Con A (Fig. 24B), or PWM (Fig. 24C).

Figures 25A, 25B, and 25C show graphs of peripheral blood lymphocyte B-cells, T-cell subpopulations, and H/S ratio, respectively, 21 days after irradiation of mice with transplanted bone marrow and treated with excipient, 40 μg IGF-I, or 120 μg IGF-I.

Figures 26A, 26B, and 26C show graphs of total splenocyte number, T-cell subpopulations and splenic B-cell number, respectively, 21 days after irradiation of mice with transplanted bone marrow and treated with excipient, 40 µg IGF-I, or 120 µg IGF-I.

Figure 27 represents a graph of the mitogenic responses 21 days after irradiation of mice with transplanted bone marrow and treated with excipient, 40 µg IGF-I, or 120 µg IGF-I using the mitogens LPS (Fig. 27A), Con A (Fig. 27B), or PWM (Fig. 27C).

Figure 28 represents a graph of thymic lymphocyte number 14 days (Fig. 28A) or 21 days (Fig. 28B) after irradiation of mice with transplanted bone marrow and treated with excipient, 40 µg IGF-I, or 120 µg IGF-I.

Description of the Preferred Embodiments

A. Definitions

As used herein, "stimulating an immune system" refers to increasing the immune function of a mammal or avian, whether the increase is due to antibody mediation or cell mediation, and whether the immune system is endogenous to the host treated with GH or GH plus IGF-I or is transplanted from a donor to the host recipient given GH or GH plus IGF-I (such as bone marrow transplants). For example, the stimulation may result from an increased number of splenic cells such as splenic lymphocyte number, splenic T-cell population number (T-cell, CD₄ and CD₈), or splenic B-cell number, or from an increased number of thymocytes. Other cells involved in the immune system response include natural killer cells, macrophages, and neutrophils. In addition, the stimulation may be due to an increase in antibody production in response to an immunogen.

As used herein, the expressions "compromised immune system" and "condition in which insufficient immunoglobulin production occurs" signify the immune system of humans as well as animals that have a smaller antibody response to antigens than normal, whether because their spleen size is smaller than it should be, whether the spleen is only partially functional, whether drugs such as chemotherapeutic agents are suppressing the normal immune function, whether the animal is functionally IGF-I (or GH) deficient, or due to any other factor. Examples include aged patients, patients undergoing chemotherapy or radiation therapy, recovering from a major illness, or about to undergo surgery, patients with AIDS, patients with congenital and acquired B-cell deficiencies such as hypogammaglobulinemia, common varied agammaglobulinemia, and selective immunoglobulin deficiencies, e.g., IgA deficiency, patients infected with a virus such as rabies with an incubation time shorter than the immune response of the patient, and patients with hereditary disorders such as diGeorge syndrome. The mammals and avians potentially affected herein include mammals and avians of economic importance such as bovine, ovine, and porcine animals, as well as chickens and turkeys. The mammals may exhibit a splenic atrophy and subsequent loss in B-cell number and function. The preferred mammal herein is a human.

As used herein, "IGF-I" refers to insulin-like growth factor from any species, including bovine, ovine, porcine, equine, avian, and preferably human, in native-sequence or in variant form, and from any source, whether natural, synthetic, or recombinant. Preferred herein for animal use is that form of IGF-I from the particular species being treated, such as porcine IGF-I to treat pigs, ovine IGF-I to treat sheep, bovine IGF-I to treat cattle, etc. Preferred herein for human use is human native-sequence, mature IGF-I, more preferably without a N-terminal methionine, prepared, e.g., by the process described in EP 230,869 published August 5, 1987; EP 128,733 published December 19, 1984; or EP 288,451 published October 26, 1988. More preferably, this native-sequence IGF-I is recombinantly produced and is available from Genentech, Inc., South San Francisco, CA for clinical investigations. Also preferred for use is IGF-I that has a specific activity greater than about 14,000 units/mg as determined by radioreceptor assay using placenta membranes, such as that available from KabiGen AB, Stockholm, Sweden.

The most preferred IGF-I variants are those described in PCT WO 87/01038 published February 26, 1987 and in PCT WO 89/05822 published June 29, 1989, i.e., those wherein at least the glutamic acid residue is absent at position 3 from the N-terminus of the mature molecule or those having a deletion of up to five amino acids at the N-terminus. The most preferred variant has the first three amino acids from the N-terminus deleted (variously designated as brain IGF, tIGF-I, des(1-3)-IGF-I, or des-IGF-I).

Recombinant human IGF-I [available commercially from KabiGen AB, Stockholm, Sweden (specific activity > 14,000 U/mg by radioreceptor assay using placental membranes) or available for clinical investigations from Genentech, Inc., South San Francisco] was employed in all the IGF-I experiments detailed in the examples. The IGF-I was dissolved at 5 mg/ml in 10 mM citrate buffer and 126 mM NaCl, pH 6.0. This IGF-I was administered to three species, i.e., rat, rabbit, and mouse, to observe its effects on spleen and thymus weight. Dose-response studies were performed in the mouse and rat, and IGF-I was given to the rabbit with similar effects. In addition, B- and T-cell numbers and responses to mitogenic stimulation were evaluated in the mice. Two animal models of GH deficiency and therefore IGF-I deficiency were used to demonstrate the effect of IGF-I on spleen and thymic weight and size. A third model of GH and IGF-I deficiency is the aged animal. Aged (18-month-old) rats were used to demonstrate the effect of IGF-I on spleen and thymic size, cellularity architecture, and *in vitro* response to mitogens. Also, adult ovariectomized rats, with normal serum IGF-I concentrations, were used to demonstrate the effect of IGF-I on spleen and thymus in an animal that was not IGF-I deficient.

As used herein, "GH" refers to growth hormone from any species, including bovine, ovine, porcine, equine, avian, and preferably human (hGH), in native-sequence or in variant form, and from any source, whether natural, synthetic, or recombinant. This includes both Met-hGH [U.S. Pat. No. 4,755,465 issued July 5, 1988 and Goeddel *et al.*, Nature, 282: 544 (1979)], which is sold under the trademark PROTROPIN® by Genentech, Inc. and is identical

to the natural polypeptide, with the exception of the presence of an N-terminal methionine residue, and recombinant hGH (rhGH), available to clinical and research investigators from Genentech, Inc. under the trademark Nutropin, and commercially available from Eli Lilly, that lacks this methionine residue and has an amino acid sequence identical to that of the natural hormone. See Gray *et al.*, *Biotechnology*, 2: 161 (1984). Both met-hGH and rhGH have equivalent potencies and pharmacokinetic values (Moore *et al.*, *supra*). Another suitable hGH candidate is an hGH variant that is a placental form of GH with pure somatogenic and no lactogenic activity. U.S. Pat. No. 4,670,393 issued 2 June 1987. Other examples of GH variants are described in WO/90/04788 published 3 May 1990. In addition to GH, other molecules which have the same activity are included within the functional meaning of growth hormone, for example fragments of GH or antibody specific for GH receptor which stimulates a GH response. In addition, any other therapeutic agent that causes a continuous expression of GH by manipulating endogenous GH secretion will fall within the functional definition of GH. For example, an increase in continuous expression of GH may be achieved by the administration of steroid hormones, for example estrogen and testosterone. The use of such agents that increases the endogenous production of GH resulting in the continuous presence of GH thereby stimulating the immune system, and other responsive tissues.

As used herein, the expression "intermittent administration" or the term "intermittent" both refer to the therapeutic use by injection, or other suitable methods, such as lung or nasal administration, of GH or GH plus IGF-I formulation in a short time interval, generally less than 60 minutes, preferably less than 30 minutes and most preferably in less than 10 minutes. There is delivery of a bolus of GH or GH plus IGF-I. This intermittent bolus delivery of the GH containing therapeutic composition may be every three or more days, preferably every 4, 5, 6 or more days, and most preferably every 7, 8, 9, 10, 12, 14, 16, 18, 21, 24 or more days.

As used herein, the expression "continuous presence" refers to a therapeutically effective plasma, serum or intracellular fluid concentration of GH or a GH variant. The GH is present in detectable amounts, at a concentration sufficient to stimulate GH responsive tissues, and the level present is not below a level the GH responsive tissues respond to as GH free.

As used herein, the expression "increasing antibody response to an immunogen" refers to raising the serum immunoglobulin (IgG, IgA or IgM) titer of an animal in response to a boost of the antigen against which the antibody is directed. Indicators of increased antibody response include an increase in the production of antibodies to booster shots of immunogen, as well as an increase in the number of B-cells in the patient. The immunogen can be any that raise antibodies directed thereto, but preferably is a virus, including a vaccine, or a bacterium. The invention is particularly useful for those instances where the mammal or avian is infected with a virus that has an incubation time that is shorter than the immune response of the mammal or avian, such as, *e.g.*, rabies. The IGF-I herein decreases the interval between primary and secondary immunizations or between secondary immunization and subsequent

boosts of immunogen. The invention is also useful in promoting immunization through the use of GH or GH plus IGF-I as hormonal adjuvants to increase both the rate of antibody production, and the amount or magnitude of the immune response. This hormonal adjuvant also increases the cellular immune response.

5 As used herein, the expression "increasing the T-cell responsiveness to an immunogen" in a subject suffering from a condition in which insufficient T-help or T-cytolytic activity occurs refers to raising the level of T-helper and/or T-cytolytic cell activity of the mammal in response to an immunogen to which T- cells are responsive, including viral antigens, tumors, bacteria, etc. A subject with insufficient T-help or T-cytolytic activity is a mammal that has
10 less than the normal number of T-helper and/or T- cytolytic cells (as determined, e.g., by CD₄/CD₈ markers) necessary to, for example, secrete antibodies, activate macrophages, and kill target cells such as virally infected or tumor cells.

As used herein, the expression "restore immunity" in a mammal means to bring the level of immunity of the mammal back to normal, whether by restoring splenic or thymic cells
15 or by increasing T- cell responsiveness or the amount of immunoglobulin produced by B- cells.

As used herein, the expression mammal refers to any mammal but especially primates, bovine, ovine, canine, feline, equine and rodentia. Specifically it includes human, cows, horses, rats, mice, rabbits, monkeys, cats, dogs and pigs.

20 **B. Modes for Carrying Out the Invention**

PEG-hGH Summary

We have made PEGylated hGH and PEGylated hGH-hGH binding protein complex and show the increased half-life in pharmacokinetic studies and higher potency in rat weight gain studies. Immunogenicity and toxicity in mice have also been studied. Random PEGylation and
25 site directed PEGylation were both used and characterized.

The present invention clearly shows that the s.c. administration of hGH as a continuous infusion or PEG-GH as daily or infrequent intermittent injections are optimal modes of GH delivery to affect the growth of the thymus and to stimulate the immune system.

PEG-GH is a particularly attractive method of practicing this invention. In one
30 example we show that only 2 injections of PEG-5 hGH 6 days apart leads to a large overgrowth of the thymus. In another example, we show that small doses of hGH given for 24 days are ineffective at stimulating thymic growth, yet the same doses of PEG-5 hGH greatly increase thymic size. Of great utility to the practice of the invention is the demonstration that to achieve optimal thymic size the PEG-5 hGH should be delivered as
35 infrequent injections, in the rat at intervals of every 6 days. Similarly for other GH dependent responses, intermittent GH administration of PEG-hGH results in superior growth stimulation. In another example we show that met-less hGH also has the desired efficacy when it is pegylated. GHBP in combination with GH results in similar stimulation of GH responses and it

may be administered intermittently. High level continuous infusion of GH will achieve similar stimulation but with the use of substantially greater amounts of GH.

The mechanism of the enhanced effect of continuous hGH on thymic growth is unknown. It is known that optimal GH responses are seen on whole body or bone growth after pulsatile GH delivery. One might therefore predict that the growth of the thymus would also be optimal with a pulsatile method of hGH administration. There are enzyme systems in the liver where different isozymes are produced depending on the pattern of GH exposure, some are optimally stimulated by continuous GH exposure. The mechanism of how the liver senses continuous or pulsatile patterns is unknown. The magnitude of this effect was quite surprising as it was possible to administer GH in different patterns that had an equal effect on whole body growth yet an almost all or none effect on the thymus.

We have shown that the thymus is restored in weight and in function by IGF-1, GH, or IGF-1 + GH administration; that this increased mass is accompanied by an increased number of thymocytes, and that the cells produced are true precursor cells for T-cells. The increased mass of the thymus, its rejuvenation in fact, found in the present invention is translated into a positive beneficial effect on the immune system as determined by antibody production, B-cell count or T-cell count.

It would be expected that in man GH would have a beneficial restorative effect on immune function in patients whose T-cells of the immune system are lacking or deficient. This invention, therefore, provides the basis for a mode of administration of met hGH, metless hGH, and particularly of PEG-5 hGH (both met hGH and metless hGH) which appears to be the most effective form of hGH at producing this response. In addition, PEG-hGH, since it can produce the response equivalent to that of a continuous mode of administration, but using infrequent injections, seems the most effective and practical way to stimulate those hGH responsive tissues that preferentially respond to continuous hGH presence.

hGH Thymus Effects

In the present invention, we made the surprising finding that the response of the thymus to GH is very dependent on the pattern of GH administration. The surprising aspect of the discovery is that the response of the thymus is optimal under a continuous exposure to GH. Other growth responses to GH, for example those of the whole body or of bone growth, previously were found optimal when GH was given in a pulsatile manner. We are unaware of any other GH-induced growth response that is optimized by a continuous GH exposure.

We first became aware of this use of GH in an experiment comparing the effects of daily GH injections and continuous GH infusions in hypox rats. The continuous GH was much more effective at stimulating thymic growth than was the same dose of GH given by daily injection. We then looked more closely at results obtained from administering PEG-GH to hypox rats. Again PEG-GH gave much greater thymic growth than did

unmodified hGH. We observed this effect with both pegylated met-hGH (protopin) and pegylated metless hGH (rhGH or nutropin).

Methods of hGH Pegylation

5 1. PEGylation with methoxypolyethylene glycol aldehyde (Me-PEG aldehyde) by reductive alkylation and purification

To 2mg/ml of hGH in PBS pH 7.0, 5mM of Me-PEG aldehyde-5000 (molecular weight 5000 dalton) and 20mM of NaCNBH₃ were added and gently mixed at room temperature for 3 hours. Ethanolamine was then added to 50mM to reductively amidate the remaining unreacted Me-PEG. The mixture was separated on anion exchange column, FPLC Mono Q. The surplus unreacted Me-PEG doesn't bind to column and can then be separated from the mixture. Two main PEGylated hGH fractions were obtained with apparent mol. wt. of 30K and 40K on reduced SDS-PAGE, vs 20K of the unreacted hGH. hGH-hGH binding protein complex was PEGylated in the same manner to give a derivative of 150kd by gel filtration.

15 2. PEGylation with N-hydroxysuccinimide PEG(NHS-PEG) and purification

To a solution containing 2mg/ml of hGH in 50 mM of Na-borate buffer at pH 8.5 or PBS at pH 7, NHS-PEG was added at a 5-fold molar excess of the total lysine concentration of hGH, and mixed at room temperature for one hour. Products were separated on Superose 12 sizing column or Mono Q of FPLC. The PEGylated hGH varied in size depending on the pH of the reaction from approximately 250K for reaction run at pH 8.5 to 40K for pH 7.0 as measured by gel filtration. The hGH-hGH binding protein complex was also PEGylated the same way with resulting mol. wt. of 400 to 600Kd from gel filtration.

20 3. PEGylation of the cysteine mutants of hGH with PEG-maleimide

A single cysteine mutant of hGH was made by site-directed mutagenesis, secreted by *E. coli* 16C9 strain and purified on an anion-exchange column. PEG-maleimide was made by reacting monomethoxy-PEG amine with sulfo-MBs in 0.1M Na-phosphate pH 7.5 for one hour at room temp and buffer changed to phosphate buffer pH 6.2. Next hGH with free extra cysteine was mixed in for one hour and final mixture was separated on Mono Q column as in Me-PEG aldehyde PEGylated hGH.

30 4. Characterization of PEGylated hGH or hGH-hGH binding protein complex

The product hGH was characterized by SDS-PAGE, gel filtration, NMR, tryptic mapping, LC-mass spectrophotometry, and *in vitro* biological assay. The extent of PEGylation was first shown by SDS-PAGE and gel filtration and then analysed by NMR, which has a specific absorption peak for the hydrogen of PEG and the number of PEG group on each molecule can be calculated. Polyacrylamide gel electrophoresis in 10% SDS was run in reduced condition and gel filtration was run on Superose 12 with PBS or 10mM Tris-HCl pH 8.0, 100mM NaCl as elution buffer. To demonstrate which residue was PEGylated, tryptic mapping was performed. PEGylated hGH was digested with trypsin at the protein/enzyme ratio of 100 to 1 in mg basis at 37°C for 4 hr in 100mM sodium acetate, 10 mM Tris-HCl, 1mM

calcium chloride, pH 8.3 and acidified to pH<4 to stop digestion before separating on HPLC Nucleosil C-18(4.6mmX150mm, 5u,100A) and the chromatogram was compared to that of non-PEGylated starting material. Each peak can then be analysed by mass spectrometry to verify the size of the fragment in the peak. We found the fragment(s) that carry PEG group usually are not retained on the HPLC column after injection and disappear from the chromatograph. Such disappearance from the chromatograph is an indication of PEGylation on that particular fragment which should contain at most one lysine residue. PEGylated hGH was assayed for its ability to bind to the hGH binding protein (hGHBP) and this binding was compared to the binding of non-PEGylated hGHBP as described (REF) as an *in vitro* biological assay.

PEGylation of hGH

The various PEGylation methods used produced mainly three kinds of PEGylated wild-type hGH, with apparent molecular weight of 30K, 40K, and 100K on reduced SDS-PAGE. Whereas in the size exclusion chromatography, the corresponding molecular weight of this three PEGylated hGH are 35K, 51K, and 250K which should be close to their native hydrodynamic volume. These were designated PEG-hGH-1, -2 and -3. From the results of the tryptic mapping, the PEG-hGH-1 and -2 both had the N-terminal 9-amino-acid fragment missing from the chromatogram and possibly pegylated, which could be confirmed by the mass spectrometry of the big molecular species found in the flow-through of the LC. From the molecular weight on SDS-PAGE, PEG-hGH-1 may have one PEG on the N-terminal amine, and PEG-hGH-2 may have two PEG on the N-terminal amine, forming a tertiary amide. The PEG-hGH-3 has about 5 PEG group per molecule based upon the NMR result, and on the tryptic map, at least 5 peptide fragments were missing, meaning they are pegylated. The sites for adding PEG groups to hGH were N-terminal Met, K38, K41, K70, K140, K145, K158 and K168. Two lysines (K) which appeared to not be pegylated were K115 and K172. Although the three pegylated hGH showed decreased binding affinity to the hGH binding protein in the *in vitro* binding assays by 2-3 fold, the rat weight gain studies surprisingly showed that they all are more potent molecules than the wild-type by at least 3 fold, which is apparently due to the increased GH serum half life. We made two kinds of pegylated hGH-hGH binding protein complex with 2 or 19 PEG per hGHBP molecule, and both showed higher potency in rat weight gain assay than hGH by itself, but not significantly higher than the non-PEGylated complex. This lack of an increase in PEG-hGH activity over hGH-hGHBP complex may be due to the complex itself already having a high enough molecule weight above the cut-off of the kidney filtration (70K) as described (Knauf, M.J. et al, J. Biol. Chem. 263, 15064-15070, 1988).

For the various purposes of this invention, the GH or GH plus IGF-I is directly administered to the mammal or avian by any suitable technique, including parenterally, and can be administered locally or systemically. The specific route of administration will depend,

e.g., on the medical history of the patient, including any perceived or anticipated side effects using IGF-I. Examples of parenteral administration include subcutaneous, intramuscular, intravenous, intraarterial, and intraperitoneal administration.

The administration of GH or GH plus IGF-I is by continuous infusion (using, *e.g.*,
5 minipumps such as osmotic pumps or insulin pumps), or by injection using, *e.g.*, intravenous intramuscular or subcutaneous means. Preferably, the administration is subcutaneous for GH or GH plus IGF-I. The administration may also be as a single bolus or by slow-release depot formulation. Most preferably, the GH or GH plus IGF-I is administered continuously by infusion or by bolus for those formulations having a long plasma half-life.

10 In addition, the GH or GH plus IGF-I is suitably administered together with any one or more of their binding proteins. Used with IGF-I for example, IGFBP-2, IGF- BP-4, or most preferably, IGFBP-3, which is described in WO 89/09268 published October 5, 1989 and by Martin and Baxter, J. Biol. Chem., 261: 8754-8760 (1986). This glycosylated protein is an acid-
15 stable component of about 53 Kd on a non-reducing SDS-PAGE gel of a 125-150 Kd glycoprotein complex found in human plasma that carries most of the endogenous IGFs and is also regulated by GH. The IGF-I is also suitably coupled to a receptor or antibody or antibody fragment for administration.

The GH or GH plus IGF-I compositions to be used in the therapy will be formulated and dosed in a fashion consistent with good medical practice, taking into account the clinical
20 condition of the individual patient (especially the side effects of treatment with GH or GH plus IGF-I), the site of delivery of the IGF-I composition, the method of administration, the scheduling of administration, and other factors known to practitioners. The "effective amount" of IGF-I for purposes herein (including an immune-stimulating effective amount) is thus determined by such considerations.

25 As a general proposition, the total pharmaceutically effective amount of the IGF-I administered parenterally per dose will be in the range of about 1 μ g/kg/day to 10 mg/kg/day of patient body weight, although, as noted above, this will be subject to therapeutic discretion. More preferably, this dose is at least 0.01 mg/kg/day, and most preferably for humans between about 0.01 and 1 mg/kg/day for the hormone. If given
30 continuously, the IGF-I is typically administered at a dose rate of about 1 μ g/kg/hour to about 50 μ g/kg/hour, either by 1-4 injections per day or by continuous subcutaneous infusions, for example, using a mini-pump. An intravenous bag solution may also be employed. The key factor in selecting an appropriate dose is the result obtained, as measured by increases in antibody production, increases in splenocyte or thymocyte number, increases in splenic B-cells,
35 *etc.*

A course of IGF-I treatment to affect the immune system appears to be optimal if continued longer than a certain minimum number of days, 7 days in the case of the mice. The length of treatment needed to observe changes and the interval following treatment for responses to occur appears to vary depending on the desired effect.

The IGF-I is also suitably administered by sustained-release systems. Suitable examples of sustained-release compositions include semi-permeable polymer matrices in the form of shaped articles, *e.g.*, films, or microcapsules. Sustained-release matrices include polylactides (U.S. Pat. No. 3,773,919, EP 58,481), copolymers of L-glutamic acid and gamma-ethyl-L-glutamate (U. Sidman *et al.*, Biopolymers, 22, 547-556 (1983)), poly(2-hydroxyethyl methacrylate) (R. Langer *et al.*, J. Biomed. Mater. Res., 15: 167-277 (1981), and R. Langer, Chem. Tech., 12: 98-105 (1982)), ethylene vinyl acetate (R. Langer *et al.*, Id.) or poly-D- (-)-3-hydroxybutyric acid (EP 133,988). Sustained-release IGF-I compositions also include liposomally entrapped IGF-I. Liposomes containing IGF-I are prepared by methods known *per se*: DE 3,218,121; Epstein *et al.*, Proc. Natl. Acad. Sci. U.S.A., 82: 3688- 3692 (1985); Hwang *et al.*, Proc. Natl. Acad. Sci. U.S.A., 77: 4030- 4034 (1980); EP 52,322; EP 36,676; EP 88,046; EP 143,949; EP 142,641; Japanese Pat. Appln. 83-118008; U.S. Pat. Nos. 4,485,045 and 4,544,545; and EP 102,324. Ordinarily, the liposomes are of the small (about 200-800 Angstroms) unilamellar type in which the lipid content is greater than about 30 mol. percent cholesterol, the selected proportion being adjusted for the optimal IGF-I therapy.

For parenteral administration, in one embodiment, the GH or GH plus IGF-I is formulated generally by mixing it at the desired degree of purity, in a unit dosage injectable form (solution, suspension, or emulsion), with a pharmaceutically acceptable carrier, *i.e.*, one that is non-toxic to recipients at the dosages and concentrations employed and is compatible with other ingredients of the formulation. For example, the formulation preferably does not include oxidizing agents and other compounds that are known to be deleterious to polypeptides.

Generally, the formulations are prepared by contacting the GH or GH plus IGF-I uniformly and intimately with liquid carriers or finely divided solid carriers or both. Then, if necessary, the product is shaped into the desired formulation. Preferably the carrier is a parenteral carrier, more preferably a solution that is isotonic with the blood of the recipient. Examples of such carrier vehicles include water, saline, Ringer's solution, and dextrose solution. Non-aqueous vehicles such as fixed oils and ethyl oleate are also useful herein, as well as liposomes.

The carrier suitably contains minor amounts of additives such as substances that enhance isotonicity and chemical stability. Such materials are non-toxic to recipients at the dosages and concentrations employed, and include buffers such as phosphate, citrate, succinate, acetic acid, and other organic acids or their salts; antioxidants such as ascorbic acid; low molecular weight (less than about ten residues) polypeptides, *e.g.*, polyarginine or tripeptides; proteins, such as serum albumin, gelatin, or immunoglobulins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids, such as glycine, glutamic acid, aspartic acid, or arginine; monosaccharides, disaccharides, and other carbohydrates including cellulose or its derivatives, glucose, mannose, or dextrans; chelating agents such as EDTA; sugar alcohols such as mannitol or sorbitol; counterions such as sodium; and/or nonionic surfactants such as polysorbates, poloxamers, or PEG.

The GH or GH plus IGF-I is typically formulated in such vehicles at a concentration of about 0.1 mg/ml to 100 mg/ml, preferably 1-10 mg/ml, at a pH of about 3 to 8. GH is generally stable at a pH of 6.5 to 8, more preferably 7.2 to 7.8. Full-length IGF-I is generally stable at a pH of no more than about 6; des(1-3)-IGF-I is stable at about 3.2 to 5. It will be understood that use of certain of the foregoing excipients, carriers, or stabilizers will result in the formation of IGF-I salts.

In addition, the IGF-I, preferably the full-length IGF-I, is suitably formulated in a suitable carrier vehicle to form a pharmaceutical composition that does not contain cells. In one embodiment, the buffer used for formulation will depend on whether the composition will be employed immediately upon mixing or stored for later use. If employed immediately, the full-length IGF-I can be formulated in mannitol, glycine, and phosphate, pH 7.4. If this mixture is to be stored, it is formulated in a buffer at a pH of about 6, such as citrate, with a surfactant that increases the solubility of the GH at this pH, such as 0.1% polysorbate 20 or poloxamer 188. The final preparation may be a stable liquid or lyophilized solid.

GH or GH plus IGF-I to be used for therapeutic administration must be sterile. Sterility is readily accomplished by filtration through sterile filtration membranes (e.g., 0.2 micron membranes). Therapeutic IGF-I compositions generally are placed into a container having a sterile access port, for example, an intravenous solution bag or vial having a stopper pierceable by a hypodermic injection needle.

GH or GH plus IGF-I or IGF-I alone ordinarily will be stored in unit or multi-dose containers, for example, sealed ampoules or vials, as an aqueous solution or as a lyophilized formulation for reconstitution. As an example of a lyophilized formulation, 10-ml vials are filled with 5 ml of sterile-filtered 1% (w/v) aqueous IGF-I solution, and the resulting mixture is lyophilized. The infusion solution is prepared by reconstituting the lyophilized IGF-I using bacteriostatic Water-for-Injection.

It is noted that hGH is stable at a higher pH than IGF-I, e.g., 7.4-7.8. When GH is administered, it is suitably administered together with one or more of its binding proteins. A well characterized such binding protein is the high-affinity growth hormone binding protein (GHBP) constituting the extracellular domain of the GH receptor that circulates in blood and functions as a GHBP in several species [Ymer and Herington, Mol. Cell. Endocrinol., 41: 153 (1985); Smith and Talamantes, Endocrinology, 123: 1489-1494 (1988); Emtner and Roos, Acta Endocrinologica (Copenh.), 122: 296-302 (1990)], including man (Baumann *et al.*, J. Clin. Endocrinol. Metab., 62: 134-141 (1986); EP 366,710 published 9 May 1990; Herington *et al.*, J. Clin. Invest., 77: 1817-1823 (1986); Leung *et al.*, Nature, 330: 537-543 (1987)). A second BP with lower affinity for GH has also been described that appears to be structurally unrelated to the GH receptor (Baumann and Shaw, J. Clin. Endocrinol. Metab., 70: 680-686 (1990)).

The doses of both GH and IGF-I can be less if used together than if IGF-I is administered alone. It is noted that practitioners devising doses of both IGF-I and GH should take into account the known side effects of treatment with these hormones. For hGH the side

EXAMPLE 1

GROWTH HORMONE STIMULATION OF RESPONSIVE TISSUES

A. Organ Weight Gain Comparing hGH Continuous Vs hGH Bolus Injection

5 In two series of experiments, the response of spleen and of thymus to continuous hGH was demonstrated.

In a first series of experiments we have used different strategies to deliver GH in a manner that causes tissues to be almost continually exposed to GH. In the first study we gave exogenous hGH by injection or by osmotic minipump. In this study the effect of injections and infusions of hGH were compared directly in hypophysectomized rats (85-105 g). The rats were anaesthetized and implanted with two subcutaneous minipumps delivering either hGH or excipient. The rats also received daily subcutaneous injections of either hGH or excipient.

There were the following groups (n=5 per group):

1-4 received excipient by Injection

- 15
- 1) hGH 5.0 mg/kg/day by pump
 - 2) hGH 1.0 mg/kg/day by pump
 - 3) hGH 0.2 mg/kg/day by pump
 - 4) hGH 0.04 mg/kg/day by pump

5-9 received excipient by Minipump

- 20
- 5) hGH 25.0 mg/kg/day by injection
 - 6) hGH 5.0 mg/kg/day by injection
 - 7) hGH 1.0 mg/kg/day by injection
 - 8) hGH 0.2 mg/kg/day by injection
 - 9) hGH 0.04 mg/kg/day by injection
- 25 10) Excipient treated control rats (excipient injections and pumps).

We found that GH given by minipump induced much greater weight gain and bone growth responses than the same doses of hGH given by injection. For example, the weight gain at 5.0mg/kg/day hGH by injection was 19.5 ± 1.7 g, compared to 32.5 ± 1.8 g by minipump, almost double the response. In addition, infusions of hGH were much more potent at stimulating spleen growth. It appears from Table I that hGH is almost 5 times as potent at stimulating splenic growth when it is delivered by minipump. These results show that the ability of hGH to stimulate immune tissues is aided by hGH delivery in a continuous manner.

30

Table I
Weight Gain and Spleen Weight Following
hGH Given By Injection or Minipump

Group	Wt Gain Injection	Wt Gain Pump	Spleen Injection	Spleen Pump
Excipient	1.9 ± 2.3		203 ± 37	
hGH 0.04	10.4 ± 2.4	10.6 ± 2.1	226 ± 18	
hGH 0.20	13.6 ± 2.5	16.8 ± 2.3	224 ± 41	264* ± 33
hGH 1.0	16.9 ± 3.1	24.8*** ± 2.4	264 ± 43	320** ± 32
hGH 5.0	19.5 ± 1.7	32.5*** ± 1.8	299 ± 22	382** ± 37
hGH 25.0	29.8 ± 2.1		389 ± 34	

Compared to same dose delivered by injection * p<0.1**p<0.01;*** p<0.001

5

In this second series of experiments the ability of a continuous hGH infusion of hGH alone, or hGH+hGHBP by minipumps to stimulate growth of the thymus relative to other organs was demonstrated. Bolus injections of hGH or hGH+hGHBP did not result in any differential preference for thymus growth.

10 35 female hypox rats (Purchased from Taconic, Germantown, New York) of 85-105 g, were weighed 4 times over 10 days to establish growth stasis (weight gain or loss of less than 7 grams). They ate pelleted lab diet and drank water ad libitum. They were group housed (5/cage) in a room controlled for lighting and temperature. They were then randomized into 5 groups of 7 rats per group based on their initial body weight. The rats were

15 then treated subcutaneously for 7 days with :

- 1) Excipient implanted pumps and excipient injections
- 2) Daily injections of rhGH (0.1 mg/kg/day)
- 3) 2) above plus GHBP (1.55 mg/kg/day) by minipump
- 4) Continuous Infusions of rhGH by osmotic minipump
- 20 5) 4) above plus GHBP (1.55 mg/kg/day) by minipump.

The hGH used was rhGH (Nutropin, Lot N9267AX, G042A) dissolved in sterile water. The GHBP (1-238) used was expressed in *E.Coli*, and dissolved in the rhGH excipient. The excipient minipumps and the excipient injections both contained the rhGH excipient. All injections (0.1 ml) were given subcutaneously in the nape of the neck. The minipumps used

25 were purchased from Alza (Alzet minipumps, Model 2001, pump rate 1.03 ul/h), and were implanted s.c. on the back under ketamine/xylazine anesthesia. The rats were sacrificed after 7 days (7 injections) and organs harvested. Group means were compared by ANOVA followed by Duncan's test. The means and standard deviations are reported.

GH induced weight gain in all 4 of the groups receiving GH, but the whole body weight gain response to GH was not different between the 4 groups, all groups gaining around 12 grams. There were small but statistically significant weight gains in the liver and kidney, but when both these organ weights were corrected or normalized to body weight they did not show a relative increase in size (the liver actually showed a statistically significant decrease in relative weight).

Therefore, the 4 GH treated groups all had similar weight gains and there was no overgrowth of the liver, spleen or the kidney. In marked contrast the thymus showed a large absolute and relative growth response, but only to continuous GH (Table II).

Table II
Weight Gains and Organs Weights in Hypox Rats

Group	Weight Gain (g)	Liver (g)	Liver % Bwt	Kidney (mg)	Kidney % Bwt	Thymus (mg)	Thymus % Bwt
1) Excipient	2.0 ± 2.3	4.33 ± 0.35	4.13 ± 0.30	707 ± 24	0.67 ± 0.03	288 ± 31	0.276 ± 0.032
2) GH injection	14.6 ± 2.3	4.75 * ± 0.29	4.04 ± 0.24	766 ± 59	0.65 ± 0.04	328 ± 33	0.279 ± 0.027
3) 2) + GHBP	12.7 ± 2.4	4.65 ± 0.09	4.00 ± 0.11	796 ± 56	0.68 ± 0.04	330 ± 46	0.284 ± 0.040
4) GH infusion	14.6 ± 1.5	4.64 ± 0.40	3.94 ± 0.32	774 ± 49	0.66 ± 0.03	401 ** ± 67	0.340 ** ± 0.055
5) 4) + GHBP	14.8 ± 2.2	4.73 * ± 0.16	3.97 ± 0.18	776 ± 62	0.65 ± 0.05	419 *** ± 45	0.352 ** ± 0.041

Compared to excipient treated rats, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

Over a 7 day period the growth response of the thymus to continuous hGH did not appear to be altered by the infusion of the hGHBP and the lack of response to the injections of hGH was not improved by the infusion of hGHBP. Based upon these results we conclude the following: 1) Over a 7-day period the small dose of hGH (0.1mg/kg/day) restored growth rate to about one third of that of normal. At this dose there was no effect of the pattern of GH administration on whole body growth (weight gain), and it did not markedly affect the growth of the "GH responsive organs", the spleen, kidney or the liver; 2) However, the mean thymus weight increased by 45% after continuous GH, but only by 14% (a statistically non-significant amount) after the same dose of GH given by injection.

Therefore, it is clear that at this dose of hGH (0.1 mg/kg/day) continuous administration and daily injection have equal effects on whole body weight gain. In contrast, the thymus shows a strong growth response only to the continuous mode of GH delivery.

In figure 1A and 1B the weight of spleen in rats receiving hGH or hGH plus GHBP for 7 days is illustrated. Measured in this series of experiments were spleen growth over 7 days in dwarf rats (dw/dw) for hGH alone at 5 doses (32, 8, 4, 2, 0.5, & 0.125 mg/kg/day) or hGH at 4 doses (8, 4, 2, 0.5, & 0.125 mg/kg/day) plus GHBP (at 2x the GH doses on a weight

basis i.e. 16, 8, 4, 1, 0.5 mg/kg/day Of GHBP). The spleen shows a huge growth response to GH + GHBP with the responses for GH and GH + GHBP being non-parallel. The high dose complex induced splenic overgrowth, in 7 days the spleen grew from 300 to 900 mg, spleens in non-GH deficient 150g rats average 500 mg. The maximum response to GH plus GHBP was greater than for GH alone. As the maximum response was different and the curves are not parallel an exact potency estimate cannot be obtained. But 2 mg/kg/day of GH + GHBP gave equivalent growth responses to 32 mg/kg/day of GH alone, suggesting a 16-fold potency difference. The non-parallelism suggests that the response to GH + GHBP is qualitatively different to that for GH alone, and that the difference could be due to the GHBP giving a more continuous GH exposure and a larger response. Clearly the rate of weight gain for hGH plus GHBP is substantially greater. This increased spleen weight gain is also plotted as a percent of total body weight (figure 1B).

B. Dose Response

55 female hypox rats (Purchased from Taconic, Germantown, New York) of 85-105 g, were weighed 4 times over 10 days to establish growth stasis (weight gain or loss of less than 7 grams). They ate pelleted lab diet and drank water ad libitum. They were group housed (5/cage) in a room controlled for lighting and temperature. They were then randomized into 11 groups of 5 rats per group based on their initial body weight. The rats were then treated for 7 days with:

- 1) Daily s.c. injections of met-hGH (0.033, 0.1, and 0.3 mg/kg/day)
- 2) Daily s.c. injections of peg-1 (0.033, 0.1, and 0.3 mg/kg/day)
- 3) Daily s.c. injections of peg-2 (0.033, 0.1, and 0.3 mg/kg/day)
- 4) Excipient.

The hGH used was met-hGH (Protropin) dissolved in sterile water. The excipient injections contained the rhGH excipient. All injections (0.1 ml) were given subcutaneously in the nape of the neck. The rats were sacrificed after 7 days (7 injections) and organs harvested. Group means were compared by ANOVA followed by Duncan's test. The means and standard deviations are reported.

hGH induced weight gain in all of the groups receiving GH, but the whole body weight gain response to both forms of the peg-GH showed roughly a 3-fold increase in potency, giving a large increase in weight gain (24 g) in the high dose PEG groups. At similar weight gains the PEG-hGH groups showed relative overgrowth of the thymus. Only the PEG treated groups showed relative organ overgrowth and the only organs showing statistically significant relative growth compared to excipient injected controls were the spleen and the thymus. The dose response curves shown in the figure 2 illustrate the non-parallel nature of this response (non-parallel compared to hGH treatment).

Therefore, at similar weight gains the long-acting PEG-hGH molecules preferentially affect the growth of the thymus. This large absolute and relative growth response may be

due to the met-hGH delivered by injections being cleared rapidly from the body whereas the PEG-hGH molecules are cleared more slowly and leads to a relative continuous GH exposure. Therefore these observations support our earlier observations, comparing minipumps and injections of hGH, that a continuous pattern of GH exposure is the preferred means of
5 delivering hGH to affect the components of the immune system.

Treatment with Peg-5 hGH for 16 days resulted in large increases in weight gain in hypophysectomized rats. The same total dose of hGH or peg-hGH was given every 1, 3 or 6 days. The greatest growth response was achieved with peg-hGH given every 6 days, the next greatest was with peg-hGH given every 3 days, followed by peg-GH every day. For
10 hGH with decreased frequency of injection the growth response declined rather than improved. Daily injection of hGH was superior to injections every 3 days followed by injections every 6 days. This unexpected improved efficacy of peg-hGH given as infrequent injections was quite unexpected as for a molecule with a prolonged half-life it would be expected that continuous or daily injections would be the optimal pattern of delivery.

15 C. hGH Administration Frequency

42 female hypox rats (Purchased from Taconic, Germantown, New York) of 85-105 g, were weighed 4 times over 10 days to establish growth stasis (weight gain or loss of less than 7 grams). They ate pelleted lab diet and drank water ad libitum. They were group
20 housed (5/cage) in a room controlled for lighting and temperature. They were then randomized into 7 groups of 6 rats per group based on their initial body weight. The rats were then treated for 24 days with:

- 1) An s.c. injection of met-hGH (0.1 mg/kg/day) every day (10 ug/injection)
- 2) An s.c. injection of met-hGH (0.1 mg/kg/day) every 3 days (30 ug/injection)
- 25 3) An s.c. injection of met-hGH (0.1 mg/kg/day) every 6 days (60 ug/injection)
- 4) An s.c. injection of peg-5 hGH (0.1 mg/kg/day) every day (10 ug/injection)
- 5) An s.c. injection of peg-5 hGH (0.1 mg/kg/day) every 3 days (30 ug/injection)
- 6) An s.c. injection of peg-5 hGH (0.1 mg/kg/day) every 6 days (60 ug/injection)
- 7) Daily s.c. injections of excipient.

30 The hGH used was met-hGH (Protropin) dissolved in sterile water. The excipient injections contained the rhGH excipient. All injections (0.1 ml) were given subcutaneously in the nape of the neck. On the days that GH injections were not given (in groups 2, 3, 5, & 6) injections of saline were given. The rats were sacrificed after 24 days and organs harvested. Group means were compared by ANOVA followed by Duncan's test. The means and
35 standard deviations are reported.

GH induced weight gain in all of the groups receiving GH, but the whole body weight gain response to the peg-GH was greatly enhanced, particularly when given as infrequent injections, and especially when given every 6 days. In marked contrast, the whole body growth response to hGH declined with less frequent injections.

At similar weight gains the PEG-hGH groups showed relative overgrowth of the thymus. Despite causing the body weight gain shown in Figure 3, injections of hGH at 0.1 mg/kg/day did not significantly increase the absolute or the relative size of the thymus. PEG-5 hGH increased absolute thymus size (by nearly 100% when given every 6 days) and also increased relative thymus size. Therefore, maximum thymus stimulation occurred with PEG-5 hGH administered every 6 days.

The results summarized in Figure 4 indicate that 0.1mg/kg/day hGH delivered as a daily s.c. injection has little effect on the thymus, yet promotes whole body growth. The increased activity of PEG-5 hGH is confirmed by this study. It would be difficult, using current technology, to routinely deliver hGH as a continuous infusion. PEG-5 hGH gives the method of the present invention a practical way of delivering GH (subcutaneous injections), in a way that the thymus responds to as continuous, yet the "continuous pattern" can be given as very infrequent injections. This approach obviously gives the administration method of the present invention a quick and effective means of being put into therapeutic practice.

15

D. MET-Less PEG-hGH Response

48 female hypox rats (purchased from Taconic, Germantown, New York) of 85-105 g, were weighed 5 times over 9 days to establish growth stasis (weight gain or loss of less than 7 grams). They ate pelleted lab diet and drank water ad libitum. They were group housed (6/cage) in a room controlled for lighting and temperature. They were then randomized into 8 groups of 6 rats per group based on their initial body weight.

Met-less rhGH (Nutropin) was pegylated by the method of Abuchowski et al. (Abuchowski, A. et al, J. Biol. Chem. 252, 3582-3586, 1977; Abuchowski, A. et al, Cancer Biochem. Biophys. 7, 175-186, 1984). Four forms of the PEG-5 rhGH were assayed, a broad pool as well as 3 pools based on arbitrary cuts of, early, mid, and late from a Sepharose-fractionating sizing column. The broad pool and the 3 pools were tested in the rats for 11 days by s.c. injections of material from the 4 batches of PEG-5 hGH.

Each rat received an injection (at 0.1 or 0.033 mg/kg/day) every sixth day (60 or 20 ug/injection). Each rat therefore only received 2 injections. The hGH used was rhGH (Nutropin) dissolved in sterile water. All injections (0.1 ml) were given subcutaneously in the nape of the neck. The rats were sacrificed after 11 days and organs harvested. Group means were compared by ANOVA followed by Duncan's test. The means and standard deviations are reported.

GH induced weight gain in all of the groups receiving GH, and the whole body weight gain response to the different PEG-5 rhGH forms was dose related. Previous results with the PEG-5 met-hGH were confirmed with rhGH as it was found that the PEG-5 forms gave large growth responses when injected every 6 days (27 and 18 g at high or low doses).

The organ weight responses were similar to those seen in the previous examples. When the weights of the liver, spleen, kidney and heart were expressed relative to body

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The organ weight responses were similar to those seen in the previous examples. When the weights of the liver, spleen, kidney and heart were expressed relative to body

weight there was no dose-related effect of the PEG-hGH forms. The only relative organ weight showing a dose response relationship was the thymus. The absolute thymic weight was greatly increased by PEG-hGH, for the broad pool (equivalent to the preparations of PEG-hGH used in the previous examples) the absolute thymus weight was increased for 60
5 μg of PEG-5 rhGH to 512 ± 83 mg, and for 20 μg of this material to 401 ± 99 mg, with the relative weights being $0.41 \pm 0.05\%$ and $0.36 \pm 0.08\%$, respectively.

Therefore, a similar organ specific selective growth response of the thymus could also be seen with pegylated met-less hGH delivered in a manner shown in the above examples to be optimally effective.

10

EXAMPLE II

Evaluation of Organ Weights, B- and T-Cell Numbers, And Response to IGF-I Mitogenic Stimulation

Recombinant human IGF-I [available commercially from KabiGen AB, Stockholm,
15 Sweden (specific activity > 14,000 U/mg by radioreceptor assay using placental membranes) or available for clinical investigations from Genentech, Inc., South San Francisco] was employed in all the IGF-I experiments detailed in the examples. The IGF-I was dissolved at 5 mg/ml in 10 mM citrate buffer and 126 mM NaCl, pH 6.0.

This IGF-I was administered to three species, *i.e.*, rat, rabbit, and mouse, to observe
20 its effects on spleen and thymus weight. Dose-response studies were performed in the mouse and rat, and IGF-I was given to the rabbit with similar effects. In addition, B- and T-cell numbers and responses to mitogenic stimulation were evaluated in the mice.

I. Rats

25 Two animal models of GH deficiency and therefore IGF-I deficiency were used to demonstrate the effect of IGF-I on spleen and thymic weight and size. A third model of GH and IGF-I deficiency is the aged animal. Aged (18-month-old) rats were used to demonstrate the effect of IGF-I on spleen and thymic size, cellularity architecture, and *in vitro* response to mitogens. Also, adult ovariectomized rats, with normal serum IGF-I concentrations, were used
30 to demonstrate the effect of IGF-I on spleen and thymus in an animal that was not IGF-I deficient.

Aged Rats

In two separate *in vivo* studies, IGF-I, GH, or IGF-I plus GH were administered for 14 days to aged 18-month-old rats to determine whether IGF-I could induce functional changes in
35 spleen and thymus in this model of thymic regression.

(i) Design

Male Fischer 344 rats of 18 months of age and 400-500 g were purchased from Harlan Sprague Dawley (HSD). These rats were bred by HSD for the NIH Institute for Aging and are the standard rat model used in aging studies. In Experiment One, 7 rats/group were

employed, and in Experiment Two, 8 rats/group. Young F344 rats (5- 8 weeks old), which were housed identically as experimental rats, were used as positive controls. The treatment groups were: (1) excipient pumps, excipient injections, (2) IGF-I pumps, excipient injections, (3) IGF-I pumps, GH injections, (4) excipient pumps, GH injections, and (5) young rats.

5 The IGF-I was loaded into two minipumps so that 1.150 mg/rat/day of IGF-I or 0.8 mg/kg/day of des-IGF-I was delivered sc as a continuous infusion. The rhGH (Nutropin brand, Genentech, Inc. formulated at 2 mg/ml in 18 mg/ml mannitol, 0.68 mg/ml glycine, and 5 mM phosphate, pH 7.4) or bGH (Monsanto) was given as a daily sc injection of 1 mg/rat/day. The excipient pump groups received identical pumps filled with the excipient for IGF-I (10 mM
10 citrate buffer and 126 mM NaCl, pH 6.0), hereinafter called "IGF-I excipient." The treatments continued for 14 days. The animals not receiving GH were injected (0.1 ml) with hGH vehicle each day.

At sacrifice, a blood sample was taken, and the liver, kidneys, heart, spleen, and thymus were removed, blotted dry, and immediately weighed. The spleen and thymus were
15 immediately placed in buffer and then cells were obtained by digestion or physical rupture. The cells were counted and then plated out at uniform density. The thymic cells were cultured with IL-1 (2 U/ml) and phytohemagglutinin (PHA) (5 µg/ml) and thymidine incorporation was measured as described by Maizel *et al.*, *J. Exp. Med.*, 153: 470-476 (1981). The spleens were similarly treated and two tests of function were performed.

20 (ii) Results

(a) Experiment One

Full-length IGF-I and rhGH were employed in this experiment. Figure 6 shows the body weight gain. After 14 days control rats had not gained weight. GH-treated rats gained 9.6 ± 11.4 g, IGF-I- treated rats gained 34.5 ± 9.4 g, and IGF-I- and GH-treated rats gained $45.5 \pm$
25 9.9 g. The response to IGF-I was clearly large, and the response to GH plus IGF-I appeared to be additive. IGF-I at the doses used was markedly anabolic. A very dramatic effect of IGF-I treatment was the large fall in blood urea nitrogen (BUN) levels from 20.7 ± 2.4 mg/dL in controls to 13.8 ± 1.8 mg/dL after IGF-I treatment; hGH had no effect. A lowered BUN indicates an anabolic metabolic state. The body weight gain data, the increased organ
30 weights, the lowered BUN, and the lowered blood enzyme levels all indicate that IGF-I was producing an anabolic state where protein synthesis was predominant over protein breakdown. The effect of IGF-I was clearly greater than that of hGH.

There was a clear effect of IGF-I on all the organ weights. Liver increased by 6.6%, kidneys by 16.6%, heart by 18.5%, thymus by 27.0%, and spleen by 80.8%. All the responses
35 were statistically significant. The only effect of hGH was to reduce liver weight significantly by 8.8%. Combined GH and IGF-I treatment did not reduce the magnitude of the effect of IGF-I on these organs, with one exception. Spleen weight was reduced for the IGF-I plus GH treatment compared to the weight of the spleen in the IGF-I alone group.

Total IGF-I levels were increased by IGF-I administration with or without concurrent hGH treatment. By itself, hGH did not significantly elevate blood total IGF-I levels.

The cells from the harvested organs were dispersed and their response to mitogens was measured. Table III shows some of the data for the thymus and spleen. The wet weight of the thymus was increased by IGF-I but not by hGH. Normal, young, 60-day-old Fischer rats were run as positive controls.

TABLE III
Cell Number in Spleen ($\times 10^8$) and Thymus ($\times 10^7$)

Group	No. Spleen Cells	No. Thymic Cells
Young Rats	2.81 \pm 0.30	4.43 \pm 0.79***
Old Rats Excipient	2.72 \pm 0.68	0.19 \pm 0.15
Old Rats IGF-1	3.58 \pm 0.86	.096 \pm 0.66**
Old Rats IGF-1 +GH	3.27 \pm 1.47	0.82 \pm 0.27***
Old Rats GH	2.50 \pm 0.51	0.36 \pm 0.28*

10 Values are Means and Standard deviations.

(Significances: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs Excipient)

15 None of the thymi from the untreated old rats yielded sufficient cells to allow full analysis in tissue culture. In contrast, 8 of the 13 rats treated with IGF-I or IGF-I plus GH did yield sufficient viable thymic cells. IGF-I treatment for 14 days caused a remarkable 5-fold increase in the number of thymic cells, although the thymus of the younger rats still contained substantially more cells.

20 Growth hormone tended to increase the number of thymic cells, but the effect (a doubling of the mean number) was not statistically significant. IGF-I plus hGH was also an effective way to increase thymic cell number. In contrast, the number of cells in the spleen was not significantly increased by IGF-I or GH treatment, although the mean values of the IGF-I-treated groups were higher. Therefore, IGF-I could increase the wet weight of the thymus and also the number of cells capable of being harvested. Then, any functional effect of the increased tissue mass and cell number was tested *in vitro* by measuring the responses of the dispersed thymocytes to mitogens, as shown in Table IV below.

TABLE IV

Thymic cells from young and old F344 rats. Untreated old rats all had insufficient thymic cells to run the assays.

Treatment	Cell No.	PHA	IL-1	PHA + IL-1
Young Rat	4.96	1764	1360	3349
Young Rat	4.80	1790	989	3836
Young Rat	3.52	2112	1462	3629
Mean		1888±193	1270±249	3604±244
Old Rats				
IGF-1	0.37	3078	672	11273
IGF-1	1.72	3524	1028	3724
IGF-1	1.68	3032	854	6532
IGF-1	1.20	1523	929	-
Mean		2789±872	870±150	7176±3815
Old Rats				
IGF-1 +GH	0.92	10436	1536	18990
IGF-1 +GH	1.06	5120	2836	17446
IGF-1 +GH	1.12	7432	2316	13429
IGF-1 +GH	0.78	5095	1796	7865
Mean		## 7020±2526	# 2121±576	## 14432 ± 4966
Old Rats				
GH	0.72	2005	581	4371
GH	0.82	11263	1780	27021
Mean	-	-	-	-

For both the PHA and IL-1 responses and their combination, the

- 5 tissue from the old rats showed a tendency toward increased activity with IGF-I alone compared to that from the younger animals, although this effect was not statistically significant. There was no additive effect of the IGF-I plus GH combination on the number of cells harvested. It was therefore surprising that IGF-I plus GH had the largest and most significant effect on all measures of thymic function. Compared to the responses of the
- 10 younger tissue, the PHA response for IGF-I plus GH was increased 3.7-fold and for the PHA plus IL-1 combination the response was increased 4-fold.

These data show that an increased mass of thymic tissue can be produced in an aged animal using IGF-I, and after the relatively short period of only 14 days of IGF-I treatment. There are previous studies in similarly aged rats that show that both GH and prolactin can

increase the size and some aspects of thymic function (Kelley, in Psychoneuroimmunology II, 2nd Ed., B. Ader et al., eds, 1990, *supra*).

It has also now been shown that the increased thymic tissue produced by IGF-I is functional tissue, in that it can respond to mitogens. There were four times as many thymic cells in the young rats, but the cells from IGF-I-treated old rats had an *in vitro* activity that was improved up to 4-fold. Therefore, according to the functional tests used, the thymus of the older rats was essentially restored to that of a much younger animal. In the thymus the effect of aging appeared to have been reversed.

(b) Experiment Two

In a second set of 18-month-old rats, a similar experiment was performed, except that bGH and des-IGF-I were employed. Also tested was the activity of des-IGF-I and whether the relatively poor effect of hGH in the first study was due to hGH antibodies (GH is very antigenic in the rat, bGH much less so).

The results are shown in Table V.

TABLE V

Blood Counts: Aged rats treated with des-IGF-1 and GH; cf Young Rats

Group	WBC	Lymphocyte No.	Hematocrit	RBC No.	MCV	Platelet No.
a Control Old	7.36 \pm 1.42	4.32 \pm 0.75	38.0 \pm 1.8	7.59 \pm 0.49	50.1 \pm 1.1	676 \pm 29
b des-IGF-1	8.12 \pm 0.76	4.23 \pm 0.41	37.8 \pm 1.8	7.29 \pm 0.38	51.8 \pm 0.5	726 \pm 69
c bGH	6.97 \pm 0.96	4.15 \pm 0.76	37.4 \pm 1.3	7.39 \pm 0.32	50.6 \pm 0.5	795 \pm 46
d des+bGH	8.93 \pm 1.90	4.80 \pm 1.16	37.6 \pm 1.2	7.01 \pm 0.22	53.9 \pm 1.6	783 \pm 98
e Young Rats	8.92 \pm 1.24	6.40 \pm 0.81	37.5 \pm 0.9	6.53 \pm 0.14	57.4 \pm 1.0	897 \pm 68

Means and Standard Deviations n=7 & 8, except for group (e) where (n=4).

The weight gains with des-IGF-I seemed less than in the first study, but were still superior to the response to bGH. The kidney and spleen showed large responses to des-IGF-I, and no significant response to GH. In general, des-IGF-I returned the blood cell counts toward those in the younger animals, with the combination of des-IGF-I and bGH being the most effective treatment. des-IGF-I tended to increase the white blood cell (WBC) and the lymphocyte number when combined with bGH. This change is similar in amount to that seen in Example IV, in man.

The results of thymic weight, cell number, and percentage of cells that were PNA (peanut agglutinin) positive are shown in Table VI.

TABLE VI

Thymus Cell Counts: Aged F344 rats treated with des-IGF-1 and bGH; cf Young Rats

Group	thymus Wt (mg)	Cell No. ($\times 10^6$)	PNA+ (%)
a Excipient Old	80 \pm 35	0.66 \pm 0.2	24 \pm 12
b des-IGF-1 (0.64)	117 \pm 27*	3.27 \pm 2.1**	72 \pm 14***
c bGH (1.0)	66 \pm 17	1.30 \pm 0.6	37 \pm 18
d des +bGH	144 \pm 39**	2.79 \pm 1.5**	69 \pm 23***
e Young Rats	338 \pm 30***	2.85 \pm 0.8**	94 \pm 2***

Means and Standard Deviations n=7 & 8, except for group e (n=4).

5 It can be seen that thymus weight was increased at sacrifice in the des-IGF-I-treated rats. This experiment was designed to test the origin and type of increased cell number in the thymus. This discrimination of the origin and type of cells was achieved by FACS analysis (described further below) using PNA as the specific marker for true thymocytes. PNA positive thymocytes are believed to be young precursor cells for T-cells.

10 The young rats had 5-fold more thymic cells than the old rats. The number of cells in the thymus was increased about 4.5-fold using des-IGF-I alone or in combination with bGH. By itself, bGH increased cell number only two-fold. These responses confirm the observations in Experiment One. The percentage of the cells that were PNA positive was unexpected. The young control rats had 95% PNA positive cells, and the aged rats only 25% positive cells.

15 Des-IGF-I by itself in these old rats increased the percentage PNA positive cells to 72% of the cells. A similar number (69%) was seen for the des-IGF-I plus bGH group. bGH by itself did not significantly affect the percentage PNA positive cells. This indicates that "real" thymic repopulation was being regenerated in the old animals, composed of precursor cells for T-cells.

20 Therefore, des-IGF-I produced a very dramatic effect by returning both the number of cells and the percentage that were PNA positive essentially to normal. IGF-I appears to have a marked effect on the rejuvenation of the thymus in an aged rat.

At sacrifice in Experiment Two in the aged rats, half the thymus was placed in 10% formalin and histological sections were prepared. The general morphology of the thymus was assessed by a veterinary pathologist as being characterized by (1) no significant lesions (the young control animals), or (2) involution (normal for the aged animals), or (3) showing evidence of lymphocytic hyperplasia. In addition, the amount of lymphocytic cellularity within the thymus was graded for all the animals, as this seemed to be the cell component that was different between the groups.

30 Using this scheme characteristic, thymic involution was seen in the excipient and the GH-treated groups. However, there was clear evidence of lymphocytic hyperplasia and the

restoration of the thymic architecture in the groups that received des-IGF-I and des-IGF-I plus bGH. The increase in the lymphocytic cellularity in the rats treated with des-IGF-I was easily distinguishable. Scoring the slides for the degree of involution and the amount of lymphocytic hyperplasia confirmed that involution was significantly reversed by des-IGF-I ($p < 0.01$,

5 Fisher's test) and that the amount of lymphocytic hyperplasia was greatly increased by des-IGF-I ($p < 0.001$). Therefore, histological examination of the thymus confirmed that IGF-I can rejuvenate the thymus of an aged animal, even where thymic involution has already occurred.

II. Rabbits

Male New Zealand White rabbits 2.0-2.5 kg were anesthetized and renal damage was induced by clamping both renal arteries for 120 minutes. At clamping, either one Alzet osmotic pump (Alza Corporation, Palo Alto, CA, Model 2ML-1) containing 2 ml of 3.3 mg des-IGF-I/ml acetic acid (100 mM, pH 4.5), or 2 Alzet osmotic pumps containing 2 ml each of 5.0 mg IGF-I/ml (in sodium chloride/sodium acetate buffer, pH 6.0) were placed in the abdominal cavity. The pumps delivered either 0.364 mg of des-IGF-I/kg/day or 1.18 mg IGF-I/kg/day for 7 days. Control animals received excipient-filled pumps. The animals were sacrificed at day 7 and the thymus and spleen were dissected.

After seven-day treatment with IGF-I the average wet weight of the thymus in IGF-I-treated rabbits ($n=6$) was 4.7 ± 0.44 g, nearly twice as large as those of the control animals (2.7 ± 0.58 g, $n=4$, $p=0.023$). When thymus size was expressed as a percentage of rabbit body weight the statistical significance of the effect increased ($p=0.014$).

After seven-day treatment with des-IGF-I, the average wet weight of the spleen in treated rabbits ($n=8$, 2.43 ± 0.44 g) was more than twice as large as that of the control rabbits ($n=7$, 1.17 ± 0.21 g, $p=0.028$).

III. Mice

The above studies using rats and rabbits established that IGF-I could cause profound changes in the immune system. The mouse was next used as a model system, as in this species immune cell markers and assays are better characterized and were readily available. Furthermore, it was desired to establish in the mouse if the effects on thymus and spleen size, cell number, and *in vitro* responses to mitogens were translated into a real functionally enhanced activity of the immune system.

Since it was shown that in aged rats IGF-I had remarkable activity in restoring the architecture and cytology of the thymus to that of a young animal and that the cells produced showed enhanced mitogenic response, aged mice were chosen as the model, in this case retired breeder male mice, which are a model of accelerated aging. The effect of IGF-I as an anabolic agent as well as an effector of immune tissue growth and function was studied in the adult aged mice. In addition, the effect of hGH and a combination of IGF-I and hGH on cell number and mitogenic stimulation was evaluated.

A. Design

1. Protocol

The following studies used retired breeder BALB/c mice 9 months old or older and weighing approximately 25 to 35 g (Harlan Sprague Dawley, San Diego, CA). Animals were housed in single cages and given food (Purina Rodent Chow 5010, St. Louis, MO) and water, *ad libitum*. All animals were weighed before being grouped into treatment groups (based on their body weight) using a randomization program. Animals were identified with stainless steel ear tags and were acclimated for at least one week.

IGF-I was administered by sc-implanted osmotic minipump (for 7-day studies, Alzet Model 2001, pump rate approximately 1 μ l/hr.; for 14-day studies, two Alzet Model 2002 minipumps, pump rate approximately 0.5 μ l/hr; Alza, Palo Alto, CA). The pumps were loaded with solution per the manufacturer's instructions, and the filled pumps were then incubated in sterile saline overnight in the refrigerator.

The pumps were filled with either the IGF-I excipient or the desired concentration of IGF-I (5 mg/ml formulated as described above), i.e., 7.5, 30, or 120 μ g IGF-I/day/7 days for 6 animals per group for the first seven-day treatment study and 120 μ g IGF-I/day/7 days for 5 animals per group for the second seven-day treatment study and the 14-day treatment study.

For hGH treatments, rhGH (Nutropin brand) was administered by itself in an amount of 9.6, 48, or 240 μ g hGH/day/14 days via two Alzet Model 2002 osmotic minipumps (0.5 μ l/hr/14 days) implanted sc to 5 animals per group, or by itself via 240 μ g hGH for 14 days via sc injection, 5 animals/group.

For combination studies of IGF-I and GH, IGF-I was administered in a dose of 120 μ g by two Alzet 2002 minipumps and GH was administered by daily sc 240- μ g injections into 5 animals/group.

2. Body and Organ Weight Determinations

The mice were anesthetized with an ip injection of approximately 0.4 ml of avertin (2,2,2-tribromoethanol and tert- amyl alcohol in phosphate buffered saline (PBS)). The dorsal scapular region was then clipped of hair and a small incision was made. A close hemostat was then inserted into the incision and pushed posteriorly. A minipump was then inserted into the pocket and the incision was closed with stainless steel wound clips, and a sc injection of 7500 U of penicillin was given. Animals were inspected daily and their body weights recorded.

Animals were sacrificed at various times following minipump placement, a large blood sample was taken, and the thymus, spleen, heart, liver, kidney, and mandibular and mesenteric lymph nodes from each treatment group were removed aseptically and weighed. The spleen, thymus, and lymph nodes were placed on ice in tissue culture media in separate vials for further assays. All data are expressed as the mean \pm standard deviation, with comparisons being made by one-way analysis of variance with follow-up comparisons being made using Duncan's Range Test.

3. Cell Preparation

The lymph nodes, spleen and thymus were dispersed using sintered glass slides to form single cell suspensions. The cells were then washed, in Eagle's minimal essential medium (MEM, Gibco, Grand Island, NY) containing 10% fetal bovine serum (FBS) (Gibco), penicillin (100 units/ml), 100 µg/ml streptomycin (Gibco), and 200/mM glutamine, and resuspended at 5 x 10⁶ viable cells/ml as determined by trypan blue dye exclusion.

B. 7 and 14 Day Studies

The purpose of these studies was to establish if IGF-I was anabolic in the intact normal mouse and if at such anabolic doses IGF-I affected thymic and splenic weight, cellularity, cell type, and responsiveness *in vitro* to mitogens. Five or six mice per group were used in these studies. On the basis of the doses known to be effective in the rat, it was decided to deliver IGF-I by continuous sc infusion at 140, 46, and 15 µg/mouse/day (approximately 4, 1.33, and 0.44 mg/kg/day).

C. Results

Effect of 7-Day Treatment

There was a dose-related effect on body weight gain over the 7 days (excipient 0.75 ± 0.75 g, low dose 0.86 ± 0.63 g, medium dose 1.31 ± 1.03 g, and high dose 3.42 ± 1.24 g), with the high-dose response being highly statistically significant compared to all other groups (p < 0.001). In the repeat experiment with the high-dose IGF-I a similar weight gain (3.55 ± 0.54 g) occurred that again was statistically greater (p < 0.001) than the gain of the excipient-treated group.

IGF-I caused significant growth of the spleen and the thymus after 7 days of treatment with IGF-I. In the first experiment there was a clear dose-related effect of IGF-I on the spleen (excipient 105 ± 14, low dose 124 ± 21; medium dose 145 ± 58; high dose 193 ± 23 mg; excipient vs. high-dose IGF-I, p < 0.001). In the repeat experiment, the spleen weight again increased (excipient 103 ± 18, high dose 206 ± 68 mg, p = 0.01). Thymus weight was unchanged in the first experiment; this was probably due to the thymus being dissected differently by different dissectors. In the repeat experiment, one dissector uniformly removed the thymus, and significant thymic growth was detected (excipient, 15.2 ± 1.3; high dose 26.2 ± 6.4 mg, p = 0.006).

The observed increase in spleen weight following seven-day treatment with 140 µg IGF-I/day was due in part to an increase in lymphocyte number. Viable lymphocytes, as determined by trypan blue exclusion, increased from 2 x 10⁸ to 5 x 10⁸ cells/spleen following 7-day treatment with IGF-I (Figure 7). This increase in cell number appeared to be due to an increase in both B- and T-cells. When B- and T-cell numbers were quantitated by FACS analyses of SIg+ and Thy 1+ cells, respectively, B-cell number increased 3 fold (1.3 x 10⁸ excipient vs. 3.5 x 10⁸ IGF-I), while T-cell number was also increased compared to controls (0.7 x 10⁷ excipients vs. 1.1 x 10⁷ IGF-I) (see figure 7).

The observed increase in thymic weight correlated with an increase in Thy 1+ thymocytes (1×10^7 excipient vs. 2.4×10^7 IGF-I). These data suggest that IGF-I has a potent mitogenic effect on lymphocyte subpopulations.

5 In contrast to the dramatic increase in lymphocyte number induced by IGF-I, the response of splenic lymphocytes to stimulation by LPS (B-cells) and Con A (T-cells) was decreased compared to controls, while the response to PWM was equivalent for both groups of mice (see Figure 7). This depressed mitogenic response suggests a lack of functional maturity in the lymphocyte population following short-term (7-day) IGF-I treatment.

Therefore, in the 7-day experiment, lymphocyte number was increased, but mitogenic
10 response was depressed.

Effect of 14-Day Treatment

Next it was determined if a longer exposure to IGF-I was required to effect lymphocyte function than was required to effect lymphocyte number. Therefore, treatment was extended to 14 days using the high dose of IGF-I (140 μ g/mouse/day). Furthermore,
15 since hGH is thought to act in part by inducing IGF-I production, the effects of hGH vs. IGF-I on lymphocyte responses were compared.

There was a significant weight gain after 14 days of treatment with IGF-I (excipient 1.49 ± 0.46 ; high dose 3.87 ± 0.45 g, $p < 0.001$). Additionally there was significant splenic growth (excipient 96 ± 12 ; high dose 163 ± 9 , $p < 0.001$), and significant thymic growth
20 (excipient 18.2 ± 4.6 ; high dose 33.8 ± 10.6 , $p = 0.017$). It can be seen that the thymus and spleen reached similar weights after 7 or 14 days of treatment.

As seen in the 7-day experiment, the spleen cell number nearly doubled (1.3×10^8 vs. 2.4×10^8) compared to controls using IGF-I treatment (Fig. 8). While there was an increase in T-cell number in the IGF-I-treated mice, the only statistically significant increase was seen in
25 the CD₄ population (3.1×10^7 vs. 4.9×10^7) (Fig. 8), suggesting that CD₄ + cells may be preferentially increased by this treatment regime. As seen in the previous experiment, IGF-I treatment resulted in substantial increases in B-cell number. IGF-I also showed an increase in T-cell number in the thymus when treatment was carried out for 14 days (see Fig. 9).

In contrast to the decreased response seen at 7 days, following 14 days of IGF-I
30 treatment the mitogenic response of splenocytes from IGF-I-treated mice was significantly elevated compared to controls (Fig. 10). These data suggest that short-term administration of IGF-I results in significant increases in lymphocyte number, but additional time is required to see alterations in lymphocyte responsiveness.

Effect of Combination After 14-Day Treatment

a. Simultaneous Treatment

35

Since hGH and IGF-I had different effects on lymphocyte populations, in the next series of experiments the effects of hGH administered simultaneously with IGF-I were examined. Whether alone or in combination with sc-injected hGH, IGF-I treatment produced

increases in total lymphocyte number in the spleen, which again appeared to be due primarily to an increase in B-cell number (Figure 11). The combination of IGF-I and hGH did have a pronounced effect on thymocyte number over IGF-I or hGH treatment alone (Figure 12).

It is expected that the preferred route of combination therapy would be administration of continuously infused IGF-I and hGH.

b. Sequential Treatment

When GH (at 280 µg/day) was administered first for 14 days followed by administration by IGF-I (at 140 µg/day) for 14 days, no effect of IGF-I was seen.

Long-Term Effects of 14-Day Treatment

To determine the long-lasting effects of IGF-I, hGH and the combination, lymphocyte populations from control and treated animals were examined 7 and 21 days after 14-day treatment with hGH, IGF-I, or the combination of IGF-I and hGH.

Seven days post-treatment the IGF-I- and IGF-I- plus hGH-treated mice had significantly elevated splenocyte numbers compared to either control, or hGH-treated mice (Fig. 13). A statistical increase in B-cell number was observed in both IGF-I-treated groups. The increase in T-cell number was significant in the IGF-I only group, but not in the combination of hGH plus IGF-I group. Furthermore, both CD₄⁺ and CD₈⁺ T-cell populations were elevated in this group compared to controls. As was the case with 14-day treatment, both groups of IGF-I-treated mice had elevated thymocyte numbers compared to hGH-treated or control mice (Fig. 14). In addition, IGF-I, alone or in combination with hGH, produced an increase in peripheral lymph node cell numbers (Fig. 16). No alteration in node T-cell number or CD₄:CD₈ ratios was observed following these treatment regimes.

Unlike the enhanced proliferative response to mitogens seen at 14 days of treatment, the mitogenic responses of the IGF-I-treated mice had returned to control values by 7 days after treatment (Fig. 15). The largest mitogenic responses were seen in the hGH- plus IGF-I-treated group compared to controls, but these differences were not statistically significant.

By 21 days after treatment, all four groups of mice had equivalent splenocyte (Fig. 17) and thymocyte (Fig. 18) numbers. Thus, 21 days appears to be sufficient to restore the normal cell number and phenotypic ratios following IGF-I treatment.

However, by 21 days after treatment, both the LPS and Con A responses of the hGH- plus IGF-I-treated group were statistically elevated compared to controls (Fig. 19). Similarly, the responses to all three mitogens were elevated in the IGF-I only group. These results suggest that IGF-I has an early and late acting effect on lymphocyte responses, while the combination of IGF-I and hGH appears to require some time to effect lymphocyte responsiveness. sc-Injected hGH alone failed to have a statistically significant effect on mitogen responses at any time point examined.

EXAMPLE III

Response to antigen in Secondary Immunization

The purpose of this experiment was to evaluate the immune function in male mice (retired breeders) immunized with dinitrophenyl-ovalbumin and treated with IGF-I. Previous experiments indicated that 14 days of continuous IGF-I administration to retired male breeder mice increased the body weight, spleen, and thymus organ weights. It was shown that the increase in spleen weight was attributable to an increase in B-cell number and an increase in mitogen responsiveness. It was also shown that increased T-cell numbers in the thymus could be generated and that these cells were also more responsive to mitogens. These data indicated that if IGF-I caused the antibody-producing B-cells and the helper T-cells to be greater in number and more responsive to mitogens, then IGF-I might be able to give a greater antibody response to an antigen.

I. Protocol

Forty-eight hours after arrival, all animals received a single ip injection (100 μ l) of dinitrophenyl-ovalbumin mixed with alum (DNPOA). (The dinitrophenyl group is a hapten that elicits a B-cell-dependent response, and the ovalbumin is a carrier that elicits a T-cell dependent response.) The DNPOA was mixed before use by adding 50 μ l of DNPOA (1 mg/ml) to 2.45 ml of sterile PBS, pH 7.0 and 2.50 ml of aluminum hydroxide absorptive gel (RehsorptarTM brand, sold by Armor Pharmaceutical Co., IL, 20 mg/ml). The DNPOA was mixed for approximately 30 minutes prior to injection. The day of DNPOA immunization is designated as Day 0.

At Day 19, ten animals were grouped by body weight into two groups. (One animal was found dead on day 9.). Nineteen mini-osmotic pumps (Alzet Corp., Palo Alto, CA) model 2002 (0.5 μ l/hr, 14 days) were filled with IGF-I excipient or IGF-I as described in Example I and placed in sterile saline solution overnight at 4°C.

At Day 20, five randomly selected animals were bled (orbitally). Serum was analyzed for IgG specific to DNPOA, as described below.

At Day 20, all ten animals were anesthetized with an ip injection of approximately 0.5 ml of avertin as described above. The animals were clipped free of hair on a dorsal area of approximately 2 cm² and wiped with 70% alcohol. A small incision, approximately 1 cm, was made in the clipped area. A hemostat was inserted into the incision and pushed anteriorly to the base of the tail and the above-described minipumps were inserted. Five animals were implanted with two minipumps each of excipient buffer. Five animals were implanted with two minipumps each of IGF-I. The rate of delivery for the minipumps gave an IGF-I dose of 120 μ g IGF-I/day for maximum of 14 days. After recovery from anesthesia, five animals each from the excipient and IGF-I groups received a booster ip 100- μ l injection of DNPOA.

At Day 25, one animal in the excipient group was found dead.

At Day 34, all nine animals were bled orbitally and the serum was analyzed for IgG (see Table VII for the overall immunization scheme).

secondary IgG response to antigen at every time point examined. While there was a trend toward elevation in total IgG levels in the IGF-I group, the values were not statistically increased compared to controls. Thus, IGF-I functions to boost the memory response of the mammal. It is noted that exposure to IgG after a secondary immunization produces a longer improvement in antibody production.

EXAMPLE IV

Effect on Immune Response After Bone Marrow Transplantation

The purpose of this experiment is to determine the effects of IGF-I treatment of mice on repopulation of the spleen and thymus following bone marrow transplantation.

I. Protocol

Male BALB/c mice, 19-26 g and 6-7 weeks old (Charles River, San Diego, CA), were used in the study. The animals were group housed in polypropylene cages with food (Purina Rodent Chow 5010, St. Louis, MO) and water, *ad libitum*. All animals were weighed the day of pump implantation and randomized into groups. Animals were identified by stainless-steel ear tags.

Ten animals per group were studied. Animals were anesthetized with an ip injection of approximately 0.4 ml of avertin prior to implantation of Alzet osmotic minipumps Model 2002 ($0.58 \pm 0.03 \mu\text{l/hr./14 days}$) filled with IGF excipient or 200 μl of rIGF-I described above diluted to achieve a daily, continuous delivery of approximately 40 or 120 $\mu\text{g/day/14 days}$.

Daily animal weights were recorded. Twenty-four hours after the implant, all animals were irradiated with 900 rads of radiation from $^{137}\text{Cesium}$ (4.29 minutes). Within one hour after irradiation animals received an intravenous injection of 1×10^7 bone marrow cells (250 μl).

Femurs and tibias were removed from 40 donor animals. The bone marrow was flushed out with PBS. Cells were centrifuged and washed with saline. Viable cells were counted and diluted with saline to achieve 10^7 cell/0.25 ml.

One half of the animals were sacrificed 14 days after the irradiation treatment. All the surviving animals from the group that was irradiated and received no bone marrow were sacrificed at this time. The remaining animals were sacrificed 23 days after the irradiation treatment. Spleens, thymuses, livers, and hearts were removed and weighed. Long bones were taken for histology and the spleens and thymuses retained for cytological and *in vitro* assays. Blood was taken for analysis of peripheral cytology. The protocol is given in Table VIII.

TABLE VIII

<u>Group</u>	<u>(n)</u>	<u>Route</u>	<u>Dose of IGF-I</u> <u>(μg/day)</u>	
1	10	sc pump	0	no marrow
2	10	sc pump	0	received marrow
3	10	sc pump	40	received marrow
4	10	sc pump	120	received marrow

II. Results**A. Weight Gain**

5 Animals not replaced with bone marrow showed a high mortality, where three out of ten animals survived for 14 days. For all measures (blood, tissue, and whole body) this group of animals showed the expected effect of a lethal dose of radiation.

Animals replaced with bone marrow survived with only two animals out of 30 dying over the 23-day study. The actual weight gains in the four groups are shown in Figure 21.

10 Thymus and spleen weights are shown in Table IX.

TABLE IX

	Thymus Weight (g)		Spleen Weight (g)	
	Day 14	Day 23	Day 14	Day 23
No marrow	8.6 \pm 0.9	-	18.6 \pm 2.5	-
Marrow only	12.6 \pm 1.0	26.0 \pm 12.9	77.8 \pm 31.5	74.0 \pm 29.0
IGF-I low	23.5 \pm 6.2	6.4 \pm 9.2	101.2 \pm 20.5	92.0 \pm 8.3
IGF-I high	27.3 \pm 10.9*	51.2 \pm 9.3**	125.0 \pm 35.4*	103.6 \pm 19.4

* $p < 0.05$ of Marrow Only on same day

** $p < 0.01$

15

There was a clear effect of IGF-I increasing thymus and spleen weight in this model. It appeared that the thymic effect was greater than the splenic effect, as there was a maintained doubling of thymus size in the high-dose IGF-I group, with the effect on the spleen initially being statistically significant, but not maintained at day 23. There was no overall effect of treatment on liver or heart weight.

20

The dramatic whole body anabolic effect of IGF-I in this setting confirms that IGF-I continues to be anabolic on the whole body. The effect of IGF-I increasing the mass of the thymus and spleen was surprising in the very extreme setting of immune deficiency that this model presents. It might be expected in other models of immune deficiency, *i.e.*, AIDS, that IGF-I would also show these remarkable efficacies.

25

The body weight changes for all four groups are shown in Figure 21. The figure shows clearly the very large weight loss in the animals following radiation exposure. There was a clear dose-related effect of IGF-I protecting the mice from this catabolism. High-dose IGF-I had a significant anabolic effect as early as seven days following treatment and this effect

persisted to the end of the study. Low-dose IGF-I also caused a significant protection at some time points ($p < 0.05$).

B. Cell Numbers and Mitogenic Responses

5 Fourteen days post irradiation, animals receiving 120 μ g IGF-I had increased numbers of CD₄⁺ T-cells in the peripheral blood compared to control or low-dose IGF-I treatment (Fig. 22). Indeed, the ratio of CD₄ to CD₈ increased from 2 to 4 in this treatment group compared to controls. These data are consistent with the preferential increases in CD₄ cells seen in the spleens of aged mice treated with IGF-I for 7 or 14 days. No effect was seen on peripheral B-cell number following IGF-I treatment.

10 When the splenic lymphocytes from these animals were quantitated by FACS analysis, IGF-I treatment was shown to produce a dose-responsive increase in the number of T- and B-cells (Fig. 23). However, no effect was seen on mitogenic responsiveness of these splenocytes when measured at this time point (Fig. 24).

As was the case with the spleen, the number of lymphocytes repopulating the thymus
15 of the IGF-I mice was increased compared to controls (Fig. 28).

When examined at 21 days post irradiation, IGF-I again induced an alteration in the peripheral blood lymphocytes CD₄:CD₈ ratio due to increases in the CD₄ + T-cell population (Fig. 25). By this time, total splenocyte numbers in the IGF-I-treated groups had returned to control values but a slight increase was still measurable in the splenic CD₄ + T cell population
20 (Fig. 26). This increase in T-cells was reflected in increased mitogenic responsiveness. Con A stimulation of splenic T-cells tripled in the high-dose IGF-I-treated mice (Fig. 27). B-cell mitogenic responses to LPS were unaffected by IGF-I treatment when examined at this time point.

Surprisingly, the thymic lymphocyte numbers of the high- and low-dose IGF-I-treated
25 mice were still dramatically increased compared to controls (Fig. 28).

Taken together with the increases in splenic CD₄ number and Con A responsiveness, these data suggest that IGF-I increases the rate of peripheral cell repopulation and supports an important therapeutic role for this molecule following syngenic bone marrow transplantation. It is anticipated that the use of GH to stimulate IGF-I production will result in
30 a similar response following syngenic bone marrow transplantation. Administration of both GH and IGF-I is expected to increase the rate and extent of such bone marrow transplantation.

EXAMPLE V**IGF-I ADMINISTRATION TO HUMANS**

This clinical investigation provides evidence that IGF-I also affects the immune system of a human.

I. Protocol

A Phase I clinical study was conducted of the safety and pharmacokinetics following repeat administration (multidose) of IGF-I in healthy adult males. Each subject of 12 human patients received a bolus injection of 0.03 mg/kg rhIGF-I as described above each morning for five consecutive days. On screening and ten hours post bolus on day five, blood samples were taken for determination of hematology.

II. Results

It was found that the hemoglobin, hematocrit, and red blood cells (RBCs) were significantly lower on day 5 as compared to screening or post-treatment week 2 ($p=0.001$, 0.0004 , 0.0005 , and 0.0005). In contrast, the white blood cells (WBCs) increased significantly from screening to day 5 (from 6.1 ± 1.5 to 7.5 ± 1.9 M/CMM, $p=0.0018$). Furthermore, at post-treatment week 2 the WBCs fell significantly from the value at day 5 (from 7.5 ± 1.9 to 6.4 ± 1.6 M/CMM, $p=0.003$), so that the pretreatment and 2-week post-treatment WBC values were not significantly different.

Therefore, despite the RBCs falling in this study, the WBCs rose. It is known that 25 to 30% of the white blood cells are lymphocytes. The 23% increase in the total number of WBCs in the blood of the IGF-I-treated subjects makes it very likely that there was also an increase in the number of lymphocytes following this course of IGF-I treatment in man. Compare Figure 22B, which shows statistically significant changes in the peripheral blood CD₄ + lymphocytes number in mice after treatment with 120 µg IGF-I. See also Table V on the increased effects of the combination of des-IGF-I and bGH on lymphocyte number and WBCs in aged rats.

CONCLUSION

Studies by Isaksson *et al.* (Acta Physiol Scand. 114:261-265, 1982) had shown that 4 daily subcutaneous injection of GH were more effective than 1 or 2 injections per day or a continuous infusion from subcutaneously implanted osmotic minipumps. On the other hand, Cotes *et al.* (J. Endocrinology 87:303-312, 1980) found that continuous s.c. infusion from minipumps was more effective than single daily injections and concluded that continuous infusion was more effective than episodic administration. Jansson, in The Sexual Dimorphism of Pulsatile Growth Hormone Secretion in Relation to Body Growth, University of Gotenburg, Gotenburg, Sweden, 1983 at page 33-35) showed that 4 s.c. injections of GH was superior to GH continuously administered by miniosmotic pumps. However, there have been no studies of the effectiveness of different patterns of GH administration on the growth of different body organs, on immunologic function.

We have discovered novel strategies for GH administration. We have shown that continuously administered GH is the preferred pattern of GH administration to stimulate GH responsive tissues, particularly the immune responsive tissue. There is a body of literature suggesting different responses to GH after either a continuous exposure or a pulsatile exposure (Clark and Rosinson, 1987). The present invention contains the first experimental results to show that immune responsive tissues prefer the continual presence of growth hormone and therefore that continuous presence of GH is the preferred method of therapeutic administration.

We have also discovered new strategies for GH administration in combination with IGF-I. To co-administer GH with IGF-I to affect immune functions we propose that GH be delivered to maintain continuous presence in the blood and intracellular fluids. The present invention establishes for the first time that GH and IGF-I have an additive effect when both are co-administered.

GH has previously been administered as a daily or every 2-day pulsatile or bolus injection route, since it was assumed and had been shown superior to short term continuous GH infusion. Surprisingly, the longer term continuous presence of GH in the plasma has been found superior for stimulating many if not most GH responsive tissues. The continuous presence of GH for 3 or more, preferably 6 or more and most preferably 14 or more days stimulates GH responsive tissue better than pulsatile administration.

GH may be continuously administered by catheter or various minipumps that deliver a constant amount of GH. However, in another aspect of the present invention, GH formulated to have a long half-life has been found to result in a superior GH response, when administered every 6 days or more. Surprisingly, the tissues and cells of the immune system have been found to respond to GH or GH plus IGF-I treatment to increase both the rate or response and the magnitude of the immune response. The intermittent administration of PEG-GH has been found to give the best GH response when administered every 6 days.

IGF-I was isolated and named first as a "somatomedin" to indicate that it mediated the whole-body growth-promoting activity of GH. It was later named IGF-I in recognition of its insulin-like metabolic activities. It is therefore surprising that IGF-I, a molecule considered to be a metabolic regulator of somatic growth, was shown to have similar growth factor activity on cells of the immune system as many of the interleukins.

It is known that GH receptors, IGF-I receptors, and insulin receptors are present on cells of the immune system. The functional effect *in vivo* of these receptors and the activity of their ligands on the immune system was unknown until the present invention. The effects of insulin and GH on the immune system have been taken to be insignificant (see, e.g., Snow, J. Immunol., 135: 776-778 (1985)). Most tissues in the body have receptors for GH, IGF-I, and insulin where these hormones act to regulate the basic metabolic functions of cells, for example, glucose uptake or amino acid transport. The receptors that have been demonstrated to be present in immune tissue could function to control these activities, rather

than act to influence their differentiation, growth, and the immunological activities. Recent literature has recognized that the role of IGF-I in affecting immune cytology or function is unknown (see Fu *et al.*, *J. Immunol.*, **146**: 1602-1608 (1991)).

It is well recognized that aged, underfed, or malnourished patients, or patients
5 suffering from illnesses or diseases, become immune deficient. It is additionally known that these patients also become IGF-I deficient. The findings herein suggest that this immune deficiency is directly related to, and exacerbated by, if not caused by, this IGF-I deficiency. Restoring IGF-I blood concentrations in patients would be expected to result in an amelioration of their immune deficiency. IGF-I dramatically affects the size of the thymus in several
10 animal models. Thymic growth has been seen in hypophysectomized and dwarf rats, in young, adult, and aged rats, in mice, and in rabbits. The thymus involutes with age in most animals; it reaches a maximal size and then begins involuting in man after puberty. This involution is associated with a decline in the activity of the immune system. This invention therefore provides in one aspect a means of stimulating the immune system of an aged human to
15 restore the thymic tissue to that of a much younger person. The combination of an agent that has anabolic activity on the major internal organs, with improvement of hematology and immune function, makes GH or GH plus IGF-I an attractive drug for treating adult or aged humans. The ability to rejuvenate the thymus and therefore boost the immune system is seen as providing a range of therapeutic opportunities.

20 Such opportunities include common varied agammaglobulinemia in which B-cells fail to mature into Ig secretory cells and the serum contains less than 250 mg/dl compared to 1000 mg/dl that is the normal concentration. IGF-I produced significant increases in serum Ig levels (Fig. 20) and may be useful in this disease.

A further use of the invention would be to administer the IGF-I to a patient who
25 suffers from a hereditary illness that results in an impaired immune system. An example of such a patient would be a child suffering from congenital thymic aplasia (DiGeorge syndrome) in which the thymus is atrophied and T-cells are severely diminished, leading to opportunistic infections that are often fatal. The reason for this disease is unknown. IGF-I might be expected to give an improved size, cellularity, and responsiveness of the thymus in these
30 patients. The course of treatment would be intermittent, with, for example, a predicted 14-day period of treatment being given followed by a resting period of more than 21 days between exposures to IGF-I. At this time, the cell counts in the immune tissues would have returned to normal, but their ability to respond to mitogens or to produce antibodies would be enhanced. Such an intermittent course of treatment of producing waves of cellular
35 development would be sustainable and lead to a long-term restoration of immune function in hereditary conditions of the DiGeorge type.

A third opportunity is acquired immunodeficiency syndrome (AIDS). Patients with AIDS have no T-cell immunity and inversed T4/T8 ratios. IGF-I has been shown to increase

T-cell mitogen responsiveness and specifically enhance CD₄ + cell number (Figs. 5, 10, 11) and as such may be a useful drug in the treatment of AIDS.

The data set forth above suggests that administration of IGF-I is beneficial to increase immunoglobulin production in patients suffering from insufficient immunoglobulin production. The interval between immunizations might be expected to be reduced by the invention herein. The more rapid proliferation of cells *in vitro* from IGF-I-treated mice suggested that enhanced antibody responses could be achieved more rapidly. This would allow more compressed immunization protocols. For example, in man it is common to give primary, secondary, and tertiary immunizations separated by many months. During this time the patient is at risk of exposure to the agent from which he or she is being protected. It would be an advantage to reduce the interval between immunizations by using IGF-I to boost the immune system so that the above risk could therefore be reduced.

Another use of the invention is to give a patient a course of IGF-I treatment during his or her recovery from major illnesses or following surgery when an infection or relapse might be expected. An enhanced immune response would be expected to aid such a patient to mount an immune challenge to the infection or relapse.

Still another use of GH or IGF-I administration is as an immunoadjuvant. Whenever immunizing a mammal or avian, priming with GH and or IGF-I to accelerate the immunization process is clearly indicated in the present invention.

In the above examples, the effectiveness of IGF-I has been demonstrated as follows: (1) in three species (mouse, rat, and rabbit); (2) in both sexes (male and female rats); and (3) in several animal models, including animals made surgically GH and IGF-I deficient (hypophysectomized rats), animals with hereditary GH and IGF-I deficiency (dwarf rats), normal animals (ovariectomized rats), normally aged animals that are IGF-I deficient (18-month-old rats), animals showing accelerated aging (retired breeder mice), and animals with reduced immune function (the aged animals).

It does not necessarily follow from the above studies that a minimum of 14 days of IGF-I treatment is needed to induce the changes observed. In the mouse 14-day treatment was chosen as this proved a reliable means of inducing immune tissue responses. It is possible that 7 days of IGF-I treatment, which did induce an increase in cell number, would eventually lead to functionally active mature lymphocytes. Additionally, less than 7 days of treatment (for example, the 5 days used in Example IV in man) might also be an effective period of administration. Furthermore, IGF-I treatment by injections rather than continuous infusion is also expected to be efficacious.

It would be reasonably expected that the rabbit, rat, and mice data herein may be extrapolated to avians, horses, cows, and other mammals, correcting for the body weight of the avian or mammal in accordance with recognized veterinary and clinical procedures. Humans are believed to respond in this manner as well. IGF-I receptors have been demonstrated on human lymphocytes [Kozak *et al.*, Cell Immunol., 109: 318 (1987)], and

evidence of similar responses in man is demonstrated in Example IV. Thus, it would be reasonably expected that in man IGF-I would have a beneficial restorative effect on immune function in all patients.

WHAT IS CLAIMED IS:

1. A method for stimulating a mammal's or avian's growth hormone responsive tissues comprising maintaining a continuous, effective plasma growth hormone concentration for a period of three or more days.
2. The method of claim 1 wherein said method is accomplished using a continuous infusion device.
3. The method of claim 1 wherein said method is accomplished using a growth hormone complexed to one or more macromolecules to reduce GH clearance from the blood plasma.
4. The method of claim 3 wherein said growth hormone is complexed with a macromolecule selected from the following: growth hormone binding protein, polyethelene glycol, polypropylene glycol and carbohydrate.
5. The method of claim 1 wherein said responsive tissues are immunoresponsive tissues.
6. The method of claim 5 wherein said growth hormone is human growth hormone.
7. The method of claim 6 wherein said human growth hormone is in combination with one or more of the following: growth hormone binding protein or IGF-I.
8. The method of claim 1 wherein said human growth hormone has covalently attached one or more polymers selected from the following: polyethelene glycol, polypropylene glycol and carbohydrate.
9. The method of claim 8 wherein said polymer is polyethelene glycol and said covalently attached polymer is polyethelene glycol attached at one or more human growth hormone amino acids selected from the following: N-terminal methionine1, lysine 38, lysine 41, lysine 70, lysine 140, lysine 145, lysine 158, and lysine 168.
10. The method of claim 1 wherein said period is for 6 or more days.
11. The method of claim 1 wherein said dose of growth hormone is from 0.01 to 10 mg/kg/day.
12. The method of claim 5 wherein the immune system is stimulated by increased splenic cell number.

13. The method of claim 5 wherein the immune system is stimulated by increased thymocyte number.
14. The method of claim 5 wherein said immuno-responsive tissues result in an increase in antibody synthesis.
15. The method of claim 5 wherein the said immuno-responsive tissues mediate cell mediated immunity.
16. The method of claim 1 wherein the mammal is a human.
17. The method of claim 16 wherein the human is an aged human.
18. The method of claim 16 wherein the human has a compromised immune system.
19. The method of claim 18 wherein the human has AIDS.
20. The method of claim 1 wherein the mammal has undergone a bone marrow transplant.
21. A method for increasing a mammal's or avian's antibody response to an immunogen comprising administering to the mammal or avian the immunogen and an effective amount of growth hormone.
22. The method of claim 21 wherein said growth hormone is administered prior to immunization.
23. The method of claim 21 wherein said growth hormone is administered simultaneously with or following immunization.
24. The method of claim 21 wherein said growth hormone is administered in combination with IGF-I.
25. The method of claim 21 wherein said mammal or avian is infected with a virus that has an incubation time that is shorter than the normal immune response of the mammal or avian.
26. The method of claim 16 wherein the administration is concurrent and wherein the immunogen is derived from a virus, tumor or a microorganism and a boost of the immunogen is given to the mammal or avian.

27. The method of claim 21 wherein the immunogen is a vaccine comprising an antigenic substance selected from the following: a virus, a bacterium, a fungus, a yeast and a tumor cell.
28. A method of increasing the T-cell responsiveness in a human or other mammalian subject in response to an immunogen, where said subject suffers from a condition in which insufficient T-helper or T-cytolytic activity occurs, comprising administering to the subject an effective amount of growth hormone, the amount being effective to increase the T-help or T-cytolytic activity.
29. The method of claim 4 wherein said growth hormone contains one or more covalently bound polyethyleneglycols.
30. The method of claim 29 wherein said growth hormone contains more than two and less than 10 polyethyleneglycols.
31. The method of claim 4 wherein said growth hormone is human growth hormone and said growth hormone binding protein is human growth hormone binding protein.
32. A human growth variant comprising native human growth hormone wherein polyethylene glycol is covalently attached to one or more of the following amino acids: Methionine 1, lysine 38, lysine 41, lysine 70, lysine 140, lysine 145, lysine 158 and lysine 168.

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FIG. 1A

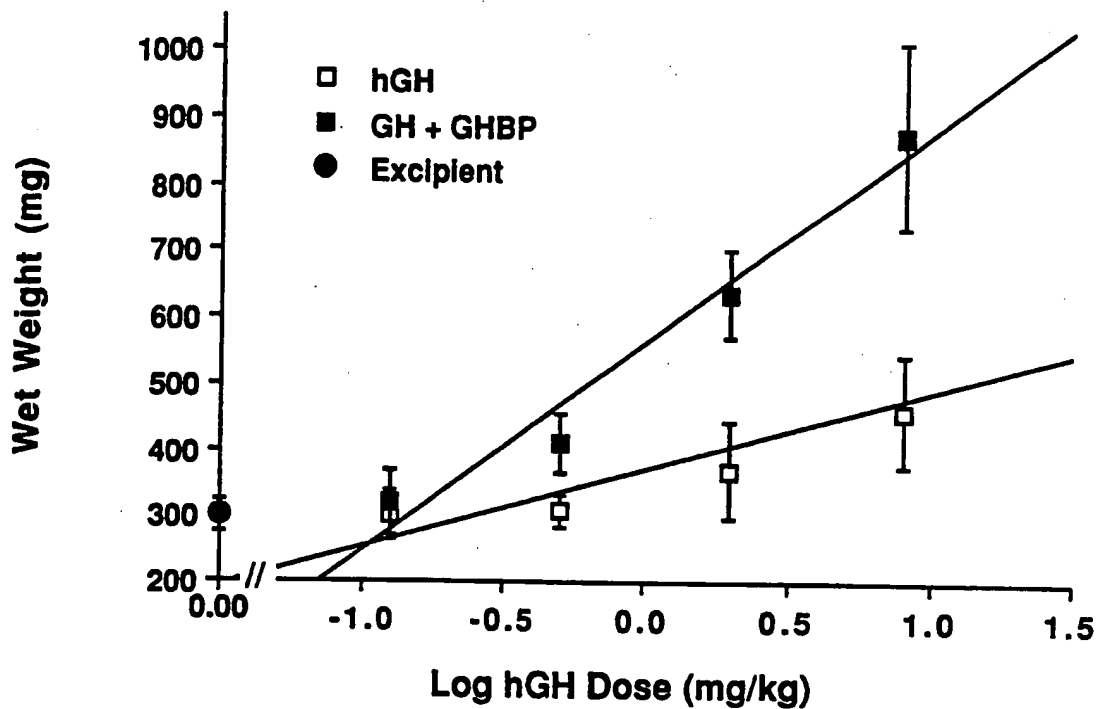
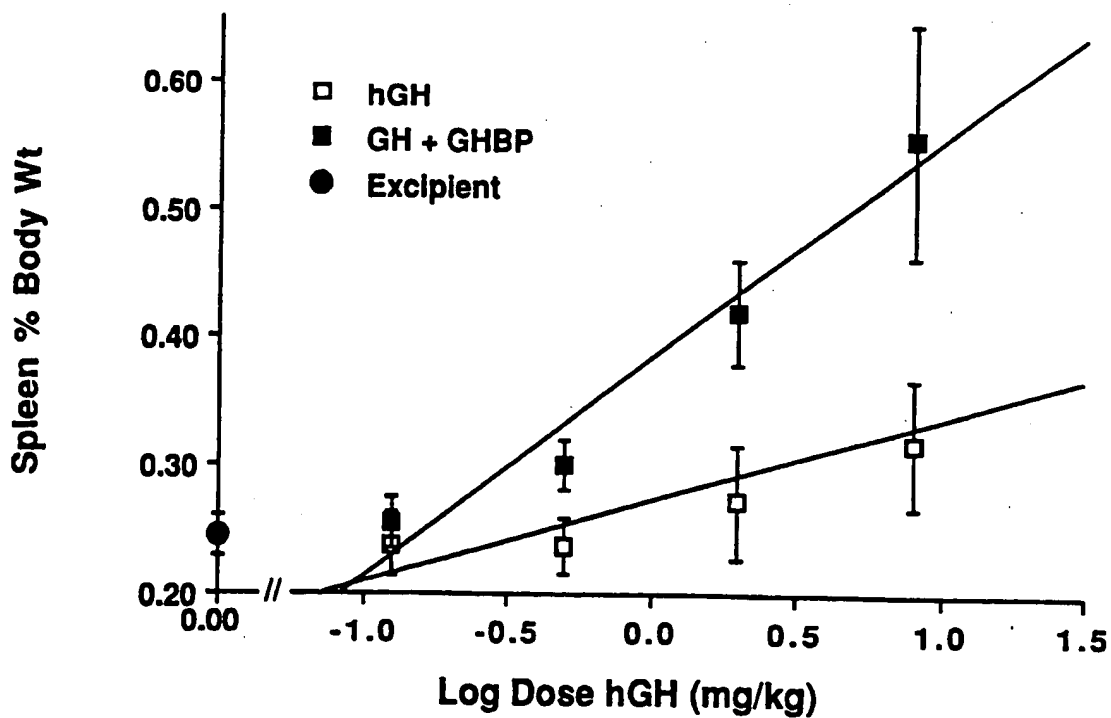


FIG. 1B



SUBSTITUTE SHEET

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FIG. 2

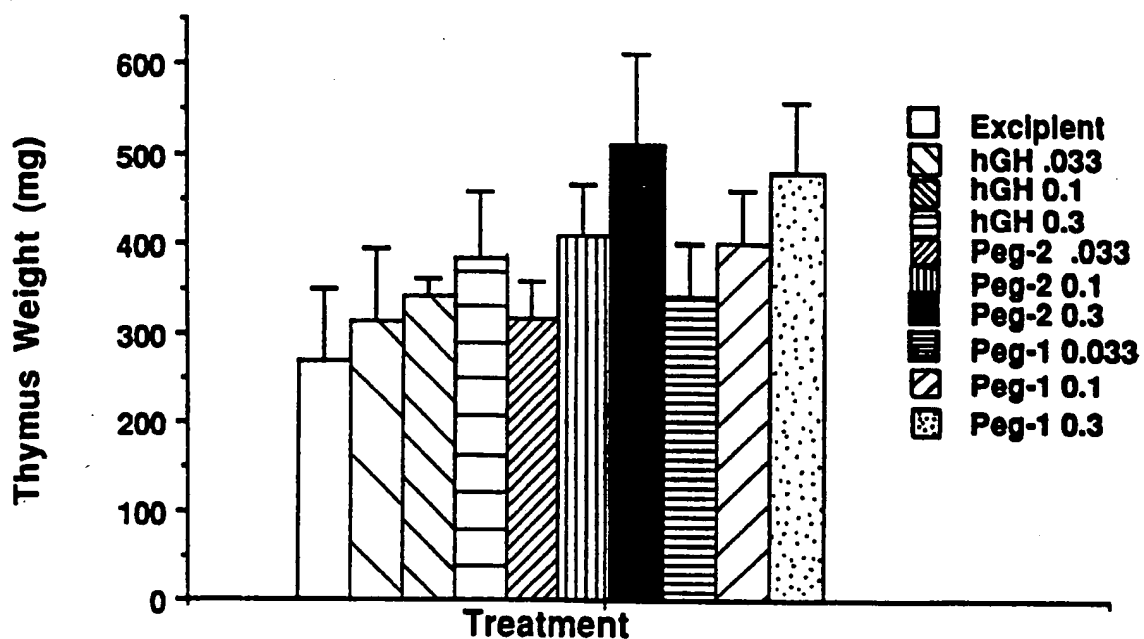
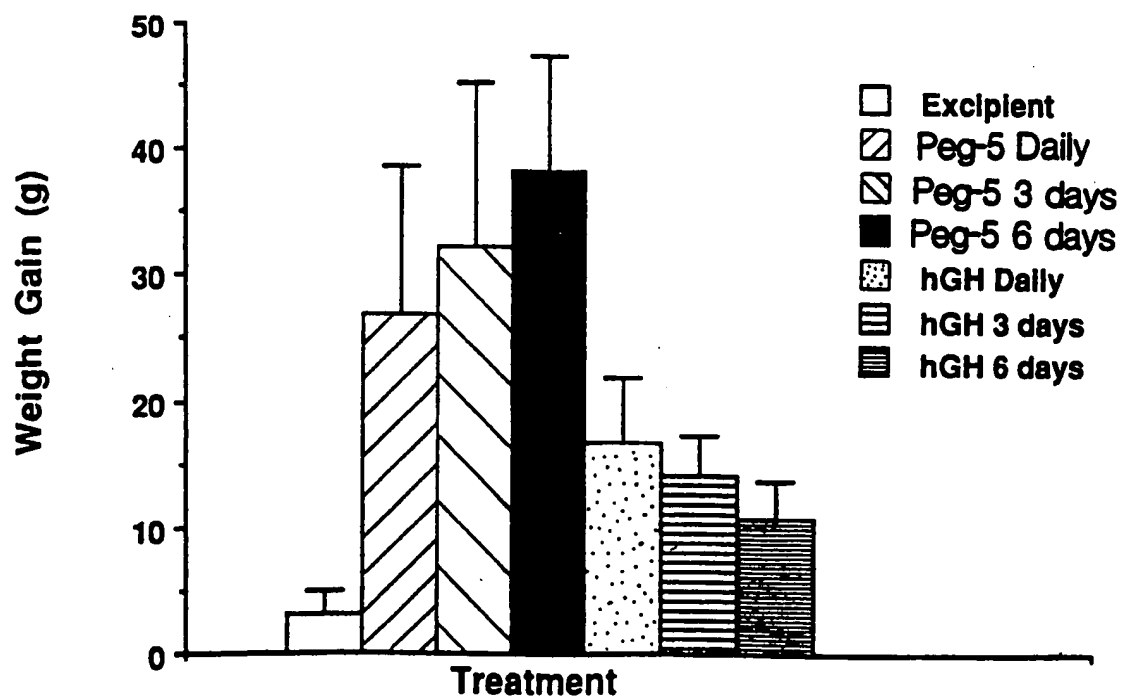


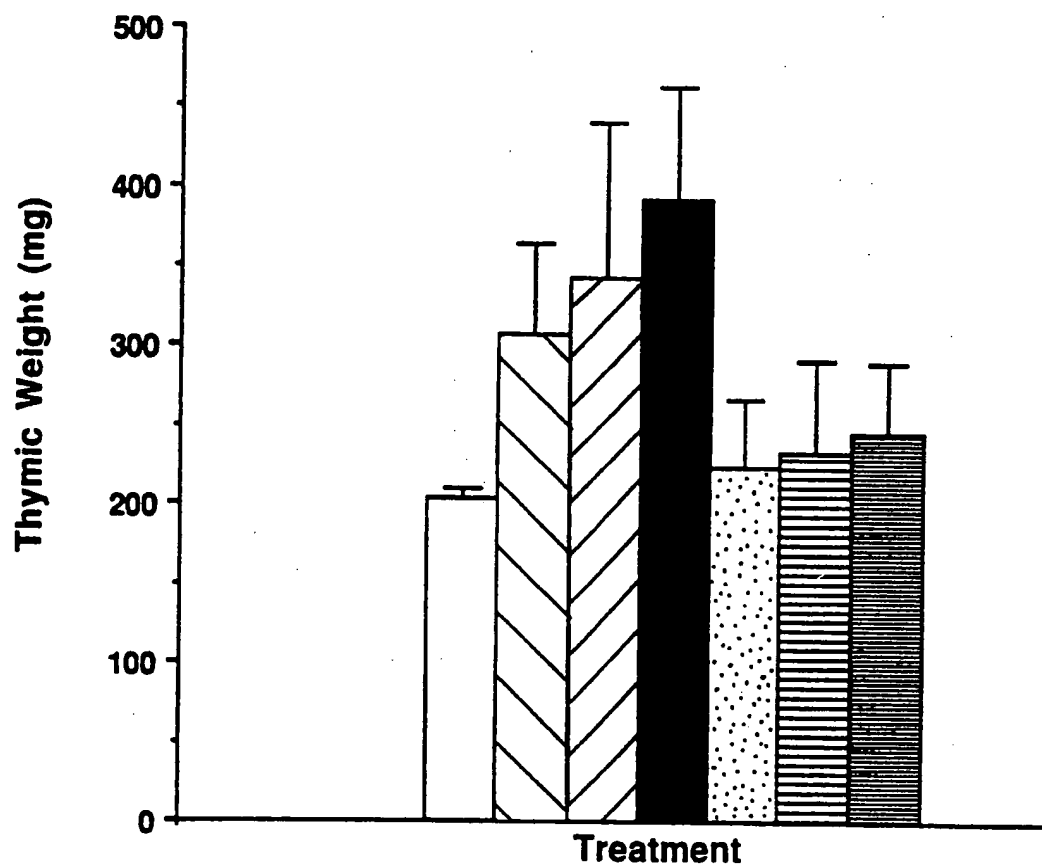
FIG. 3



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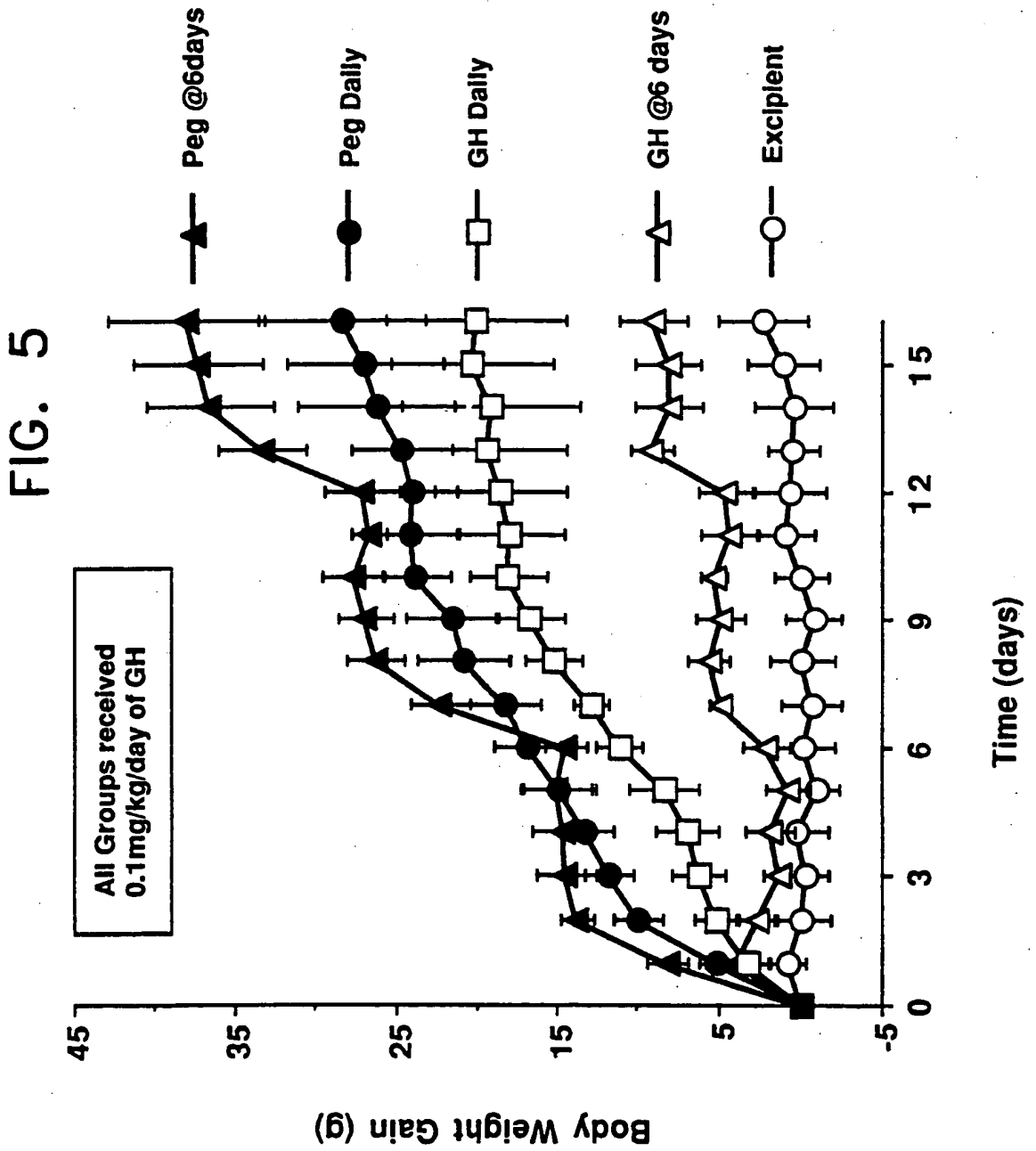
FIG. 4



- Excipient
- ▧ Peg Daily
- ▨ Peg 3 days
- Peg 6 days
- ▩ hGH Daily
- ▨ hGH 3 days
- ▨ hGH 6 days

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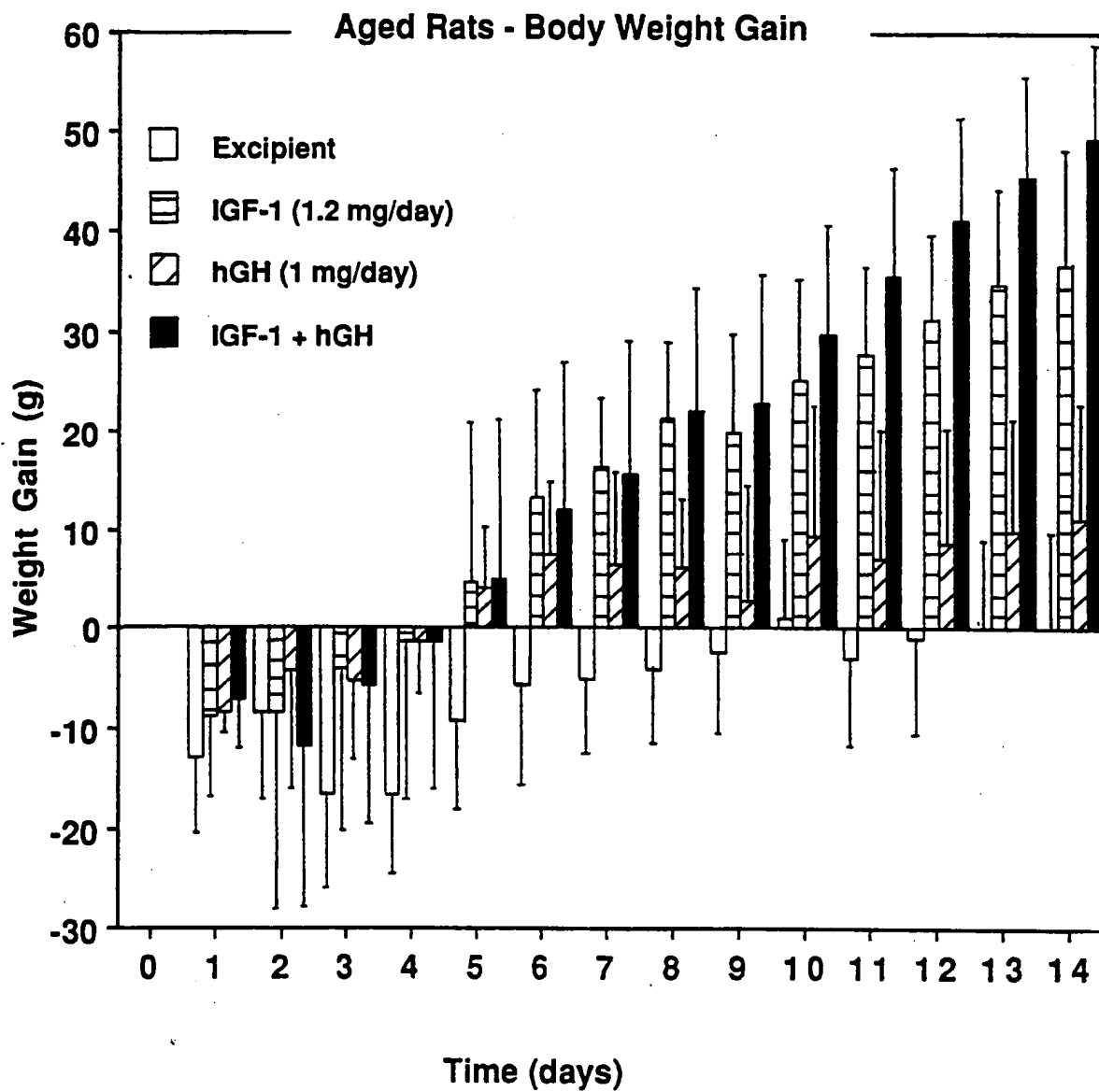


FIG. 6

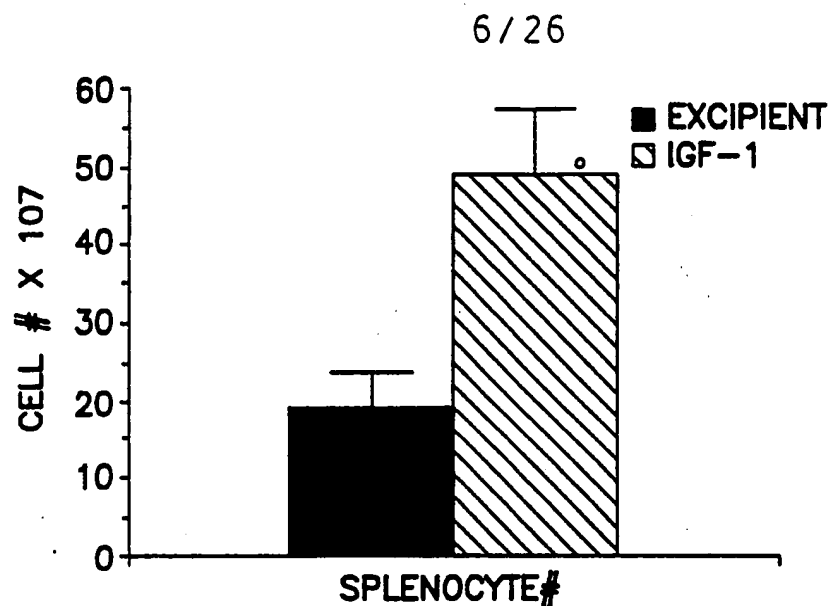


FIG. 7A

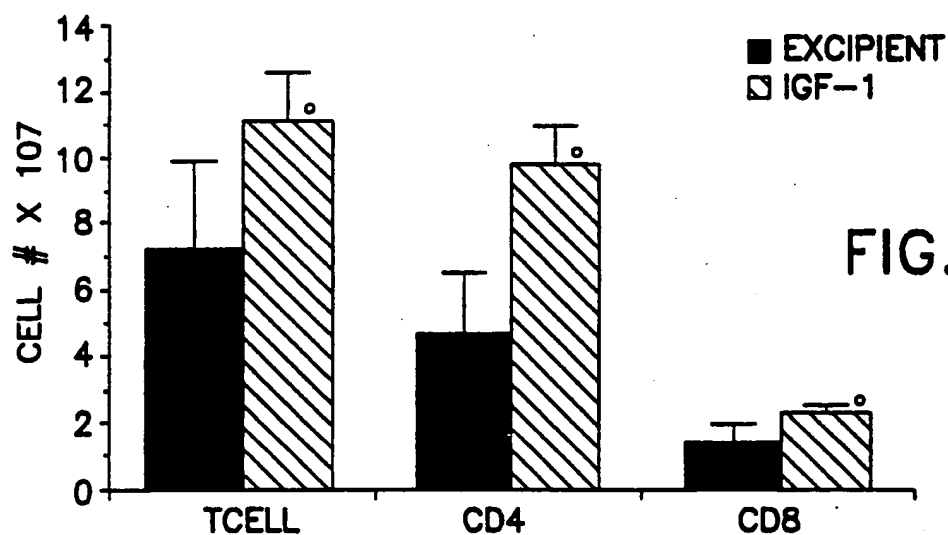


FIG. 7B

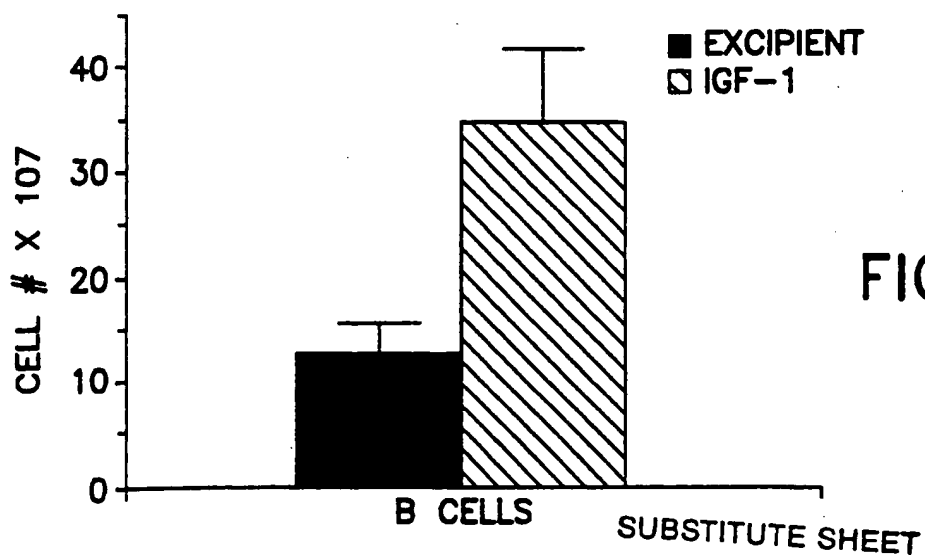


FIG. 7C

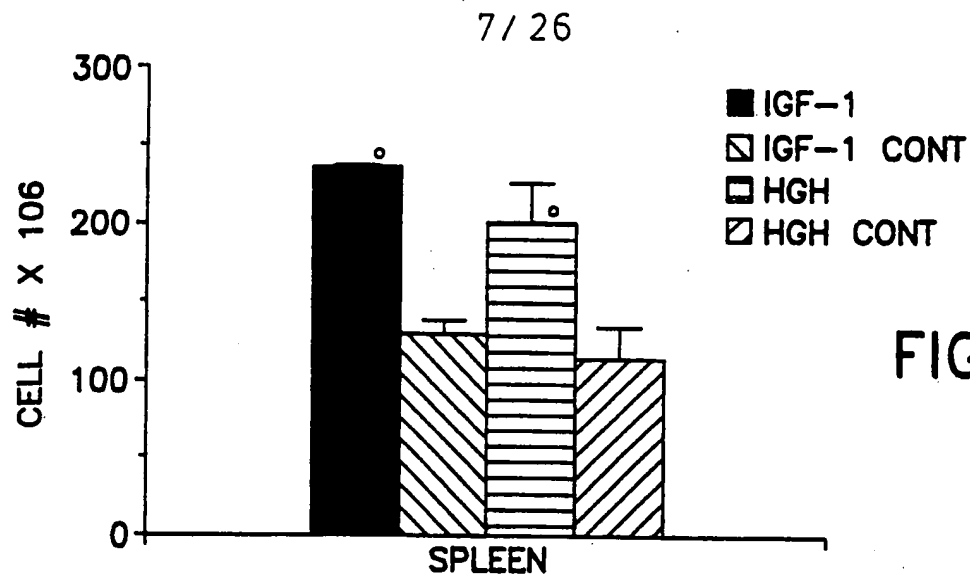


FIG. 8A

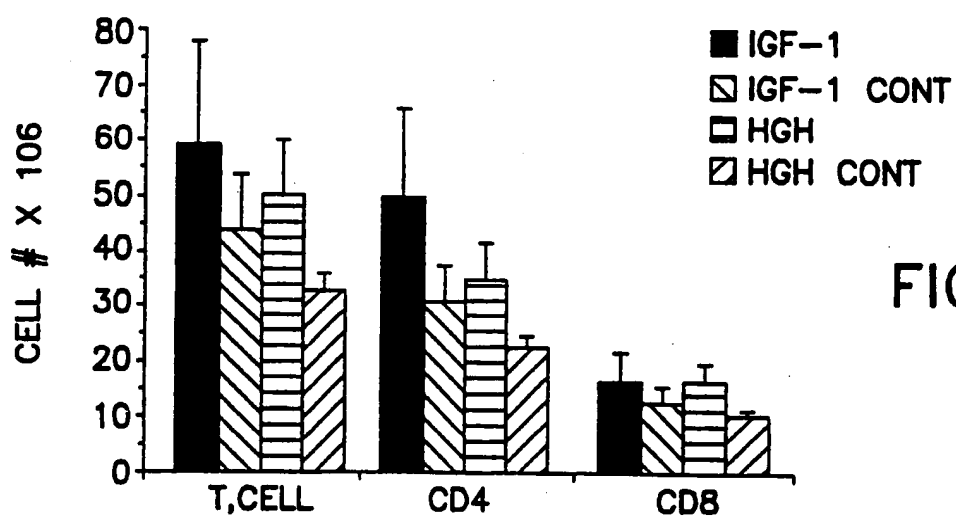


FIG. 8B

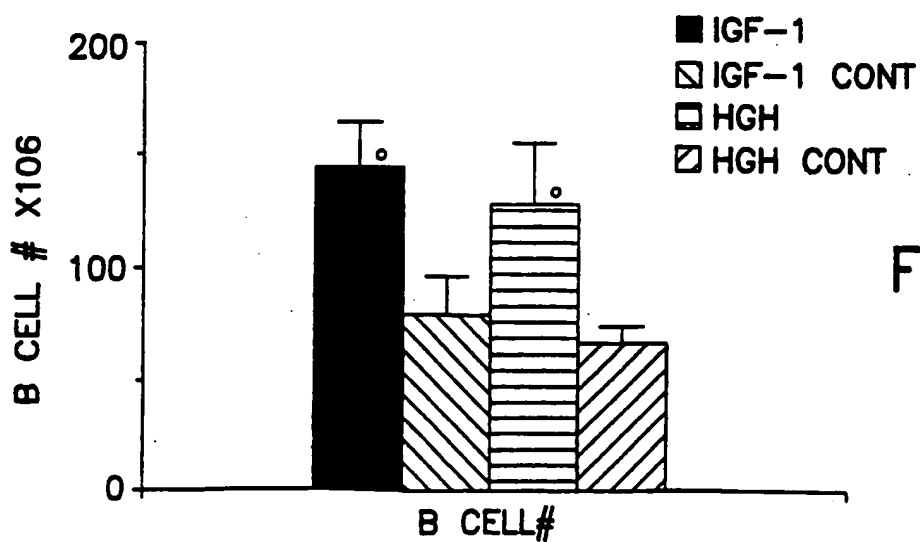


FIG. 8C

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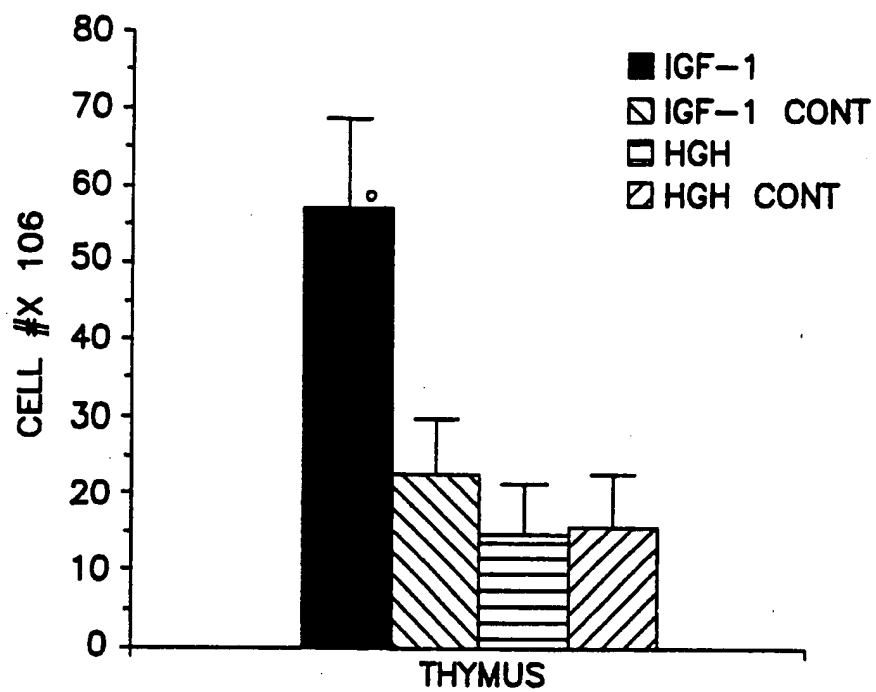


FIG. 9

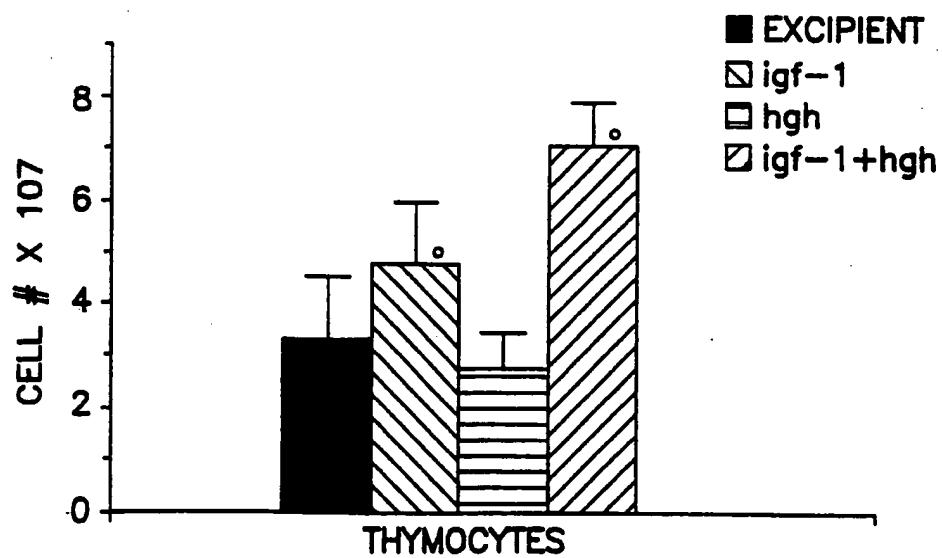
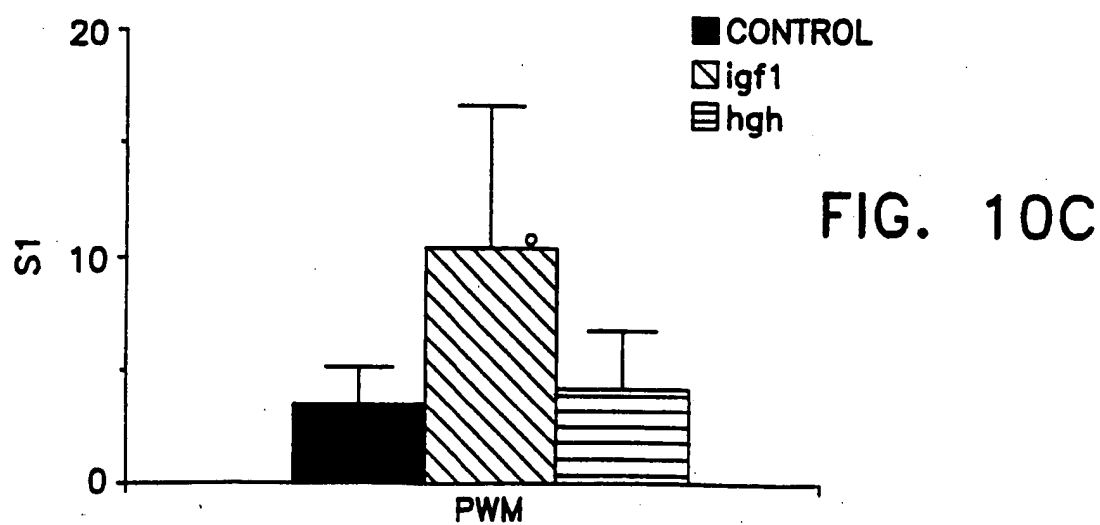
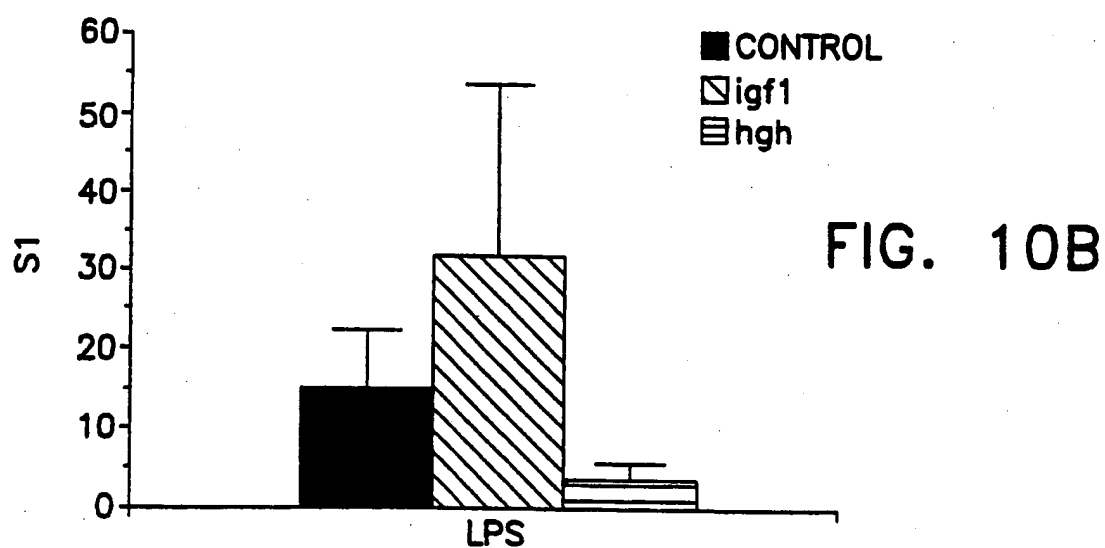
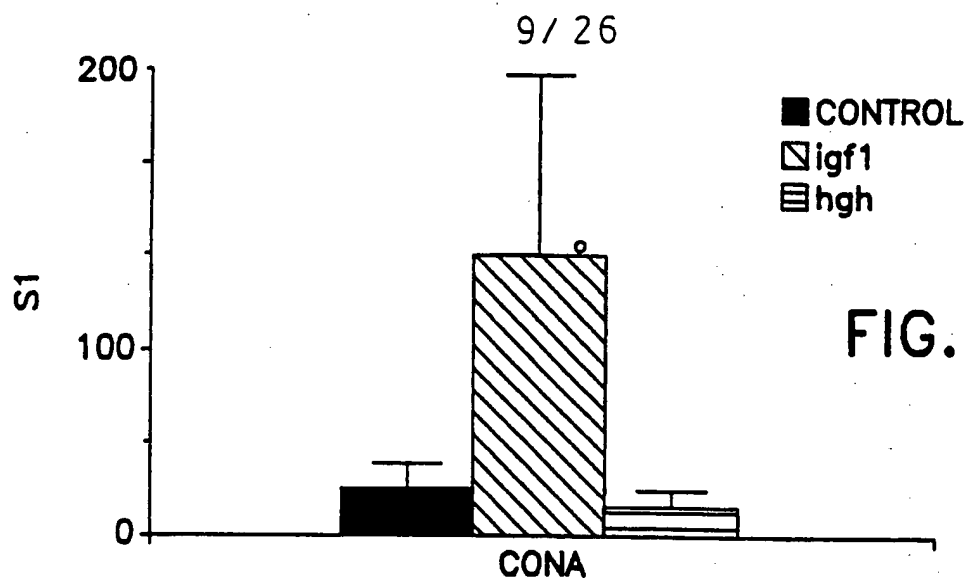


FIG. 12



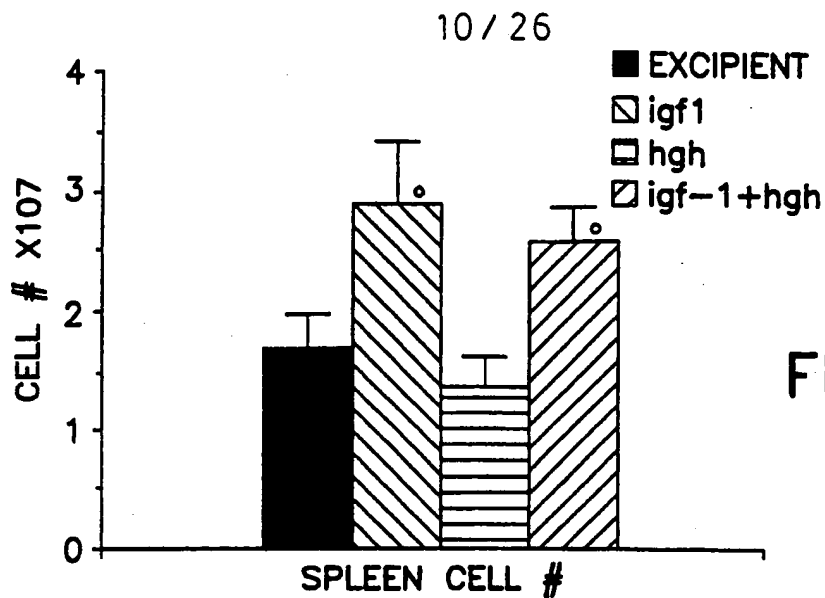


FIG. 11A

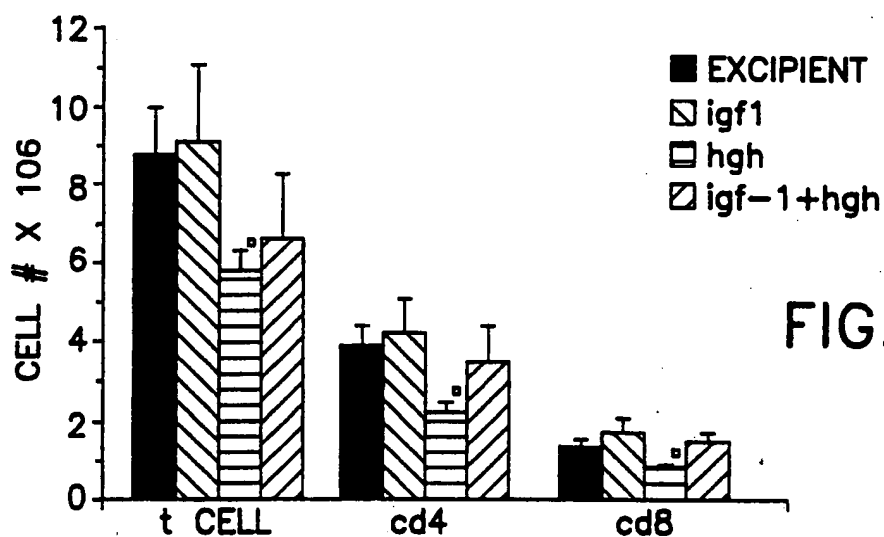


FIG. 11B

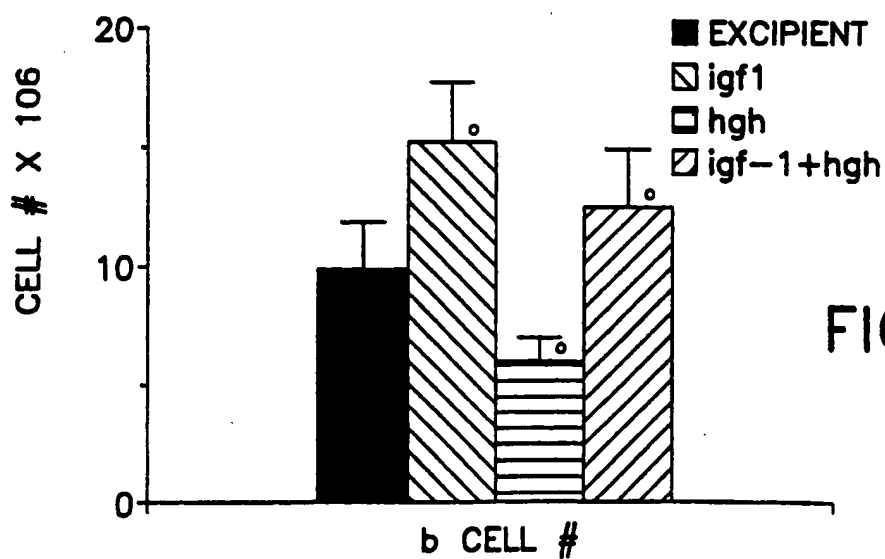


FIG. 11C

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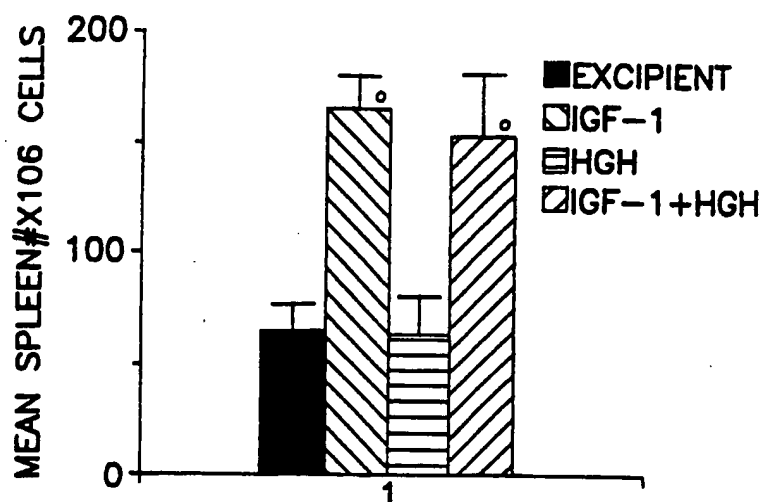


FIG. 13A

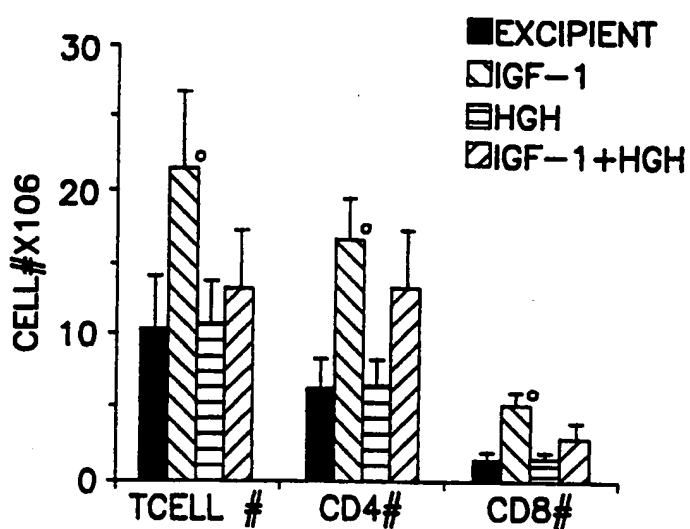


FIG. 13B

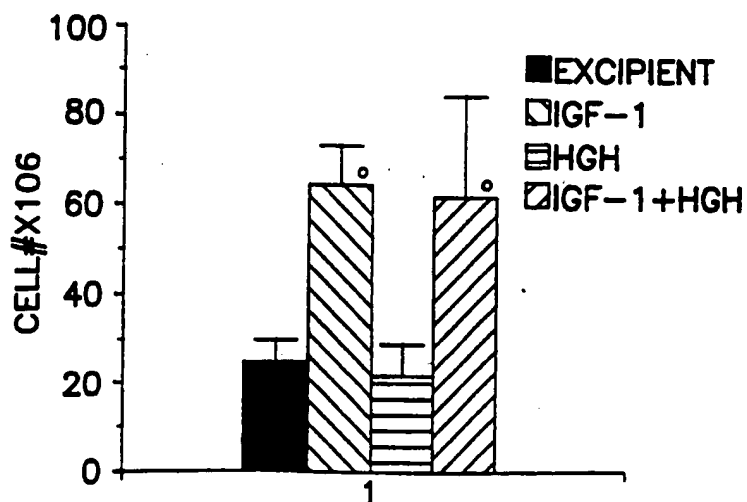


FIG. 13C

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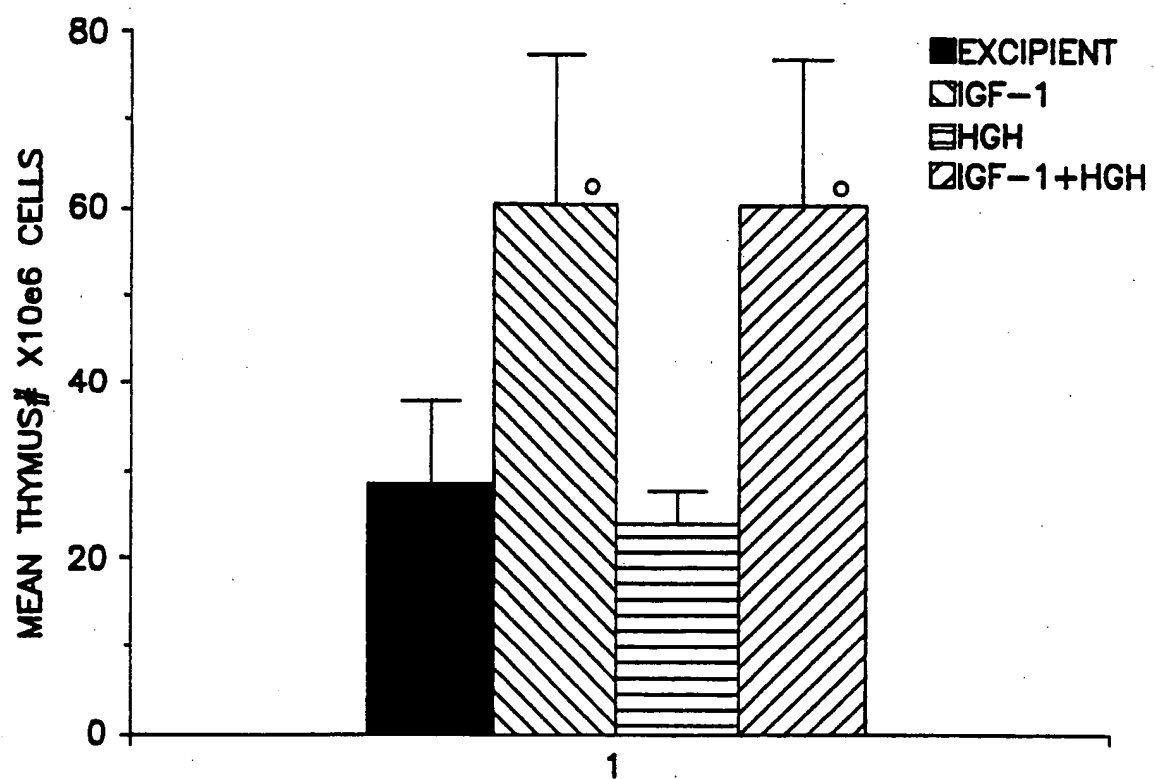


FIG. 14

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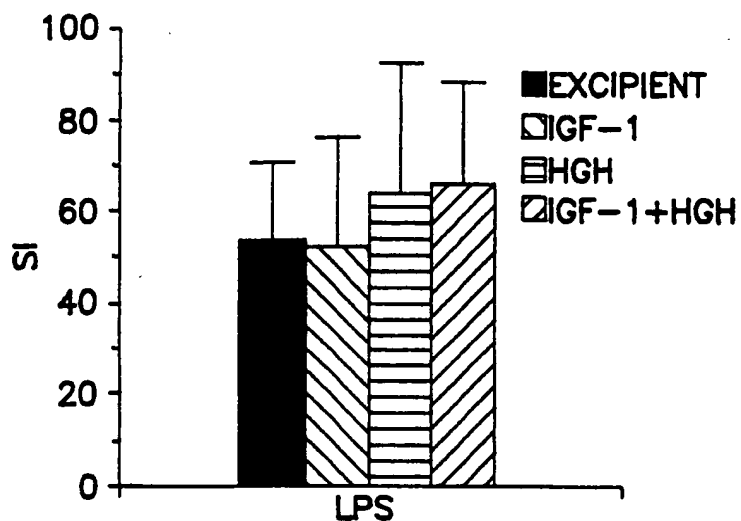


FIG. 15A

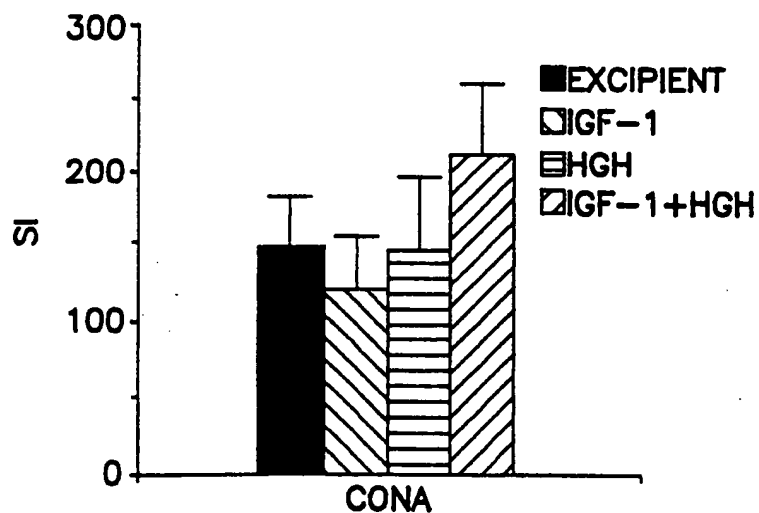


FIG. 15B

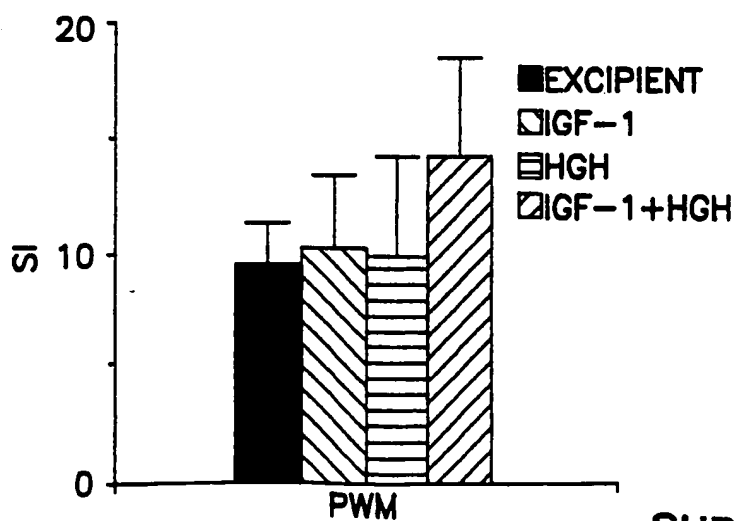


FIG. 15C

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FIG. 16A

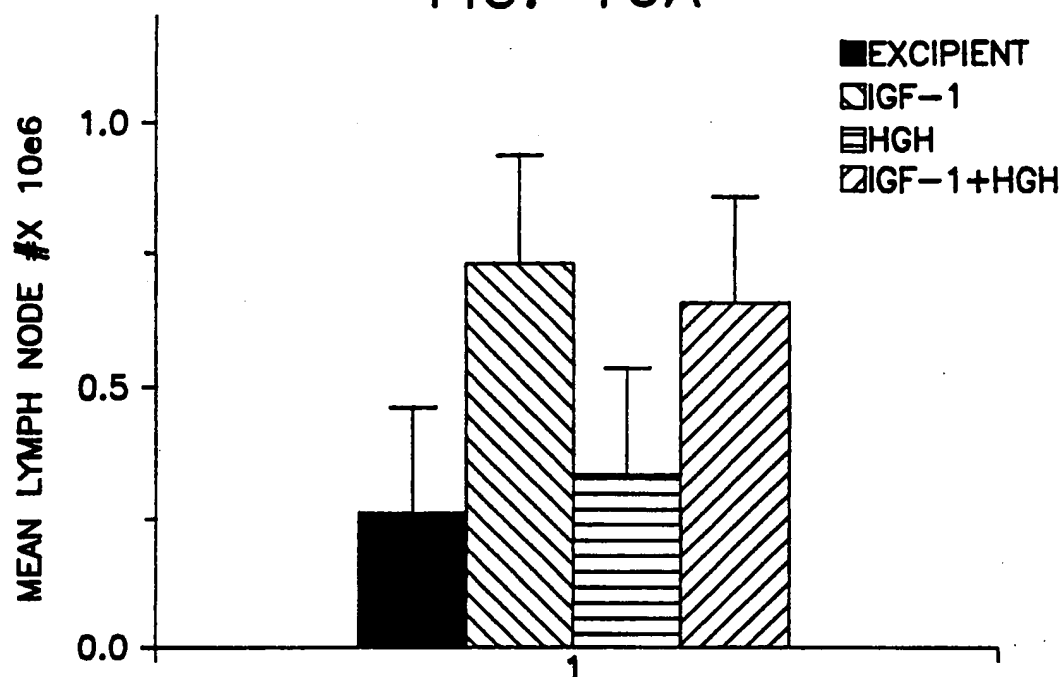
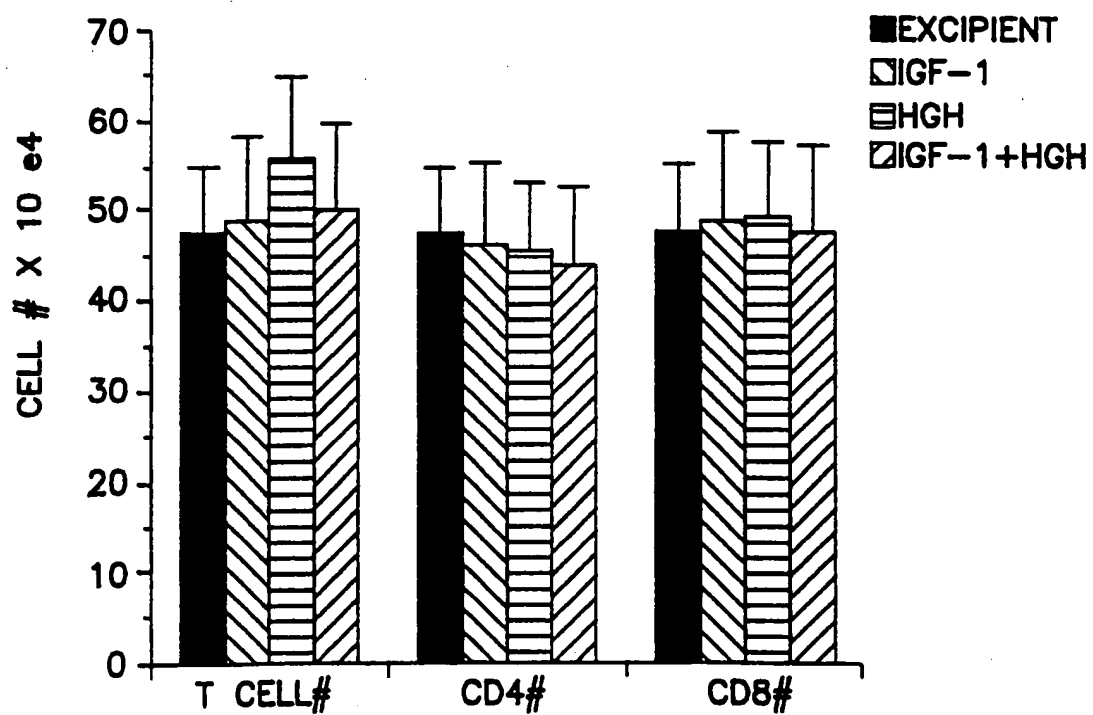


FIG. 16B



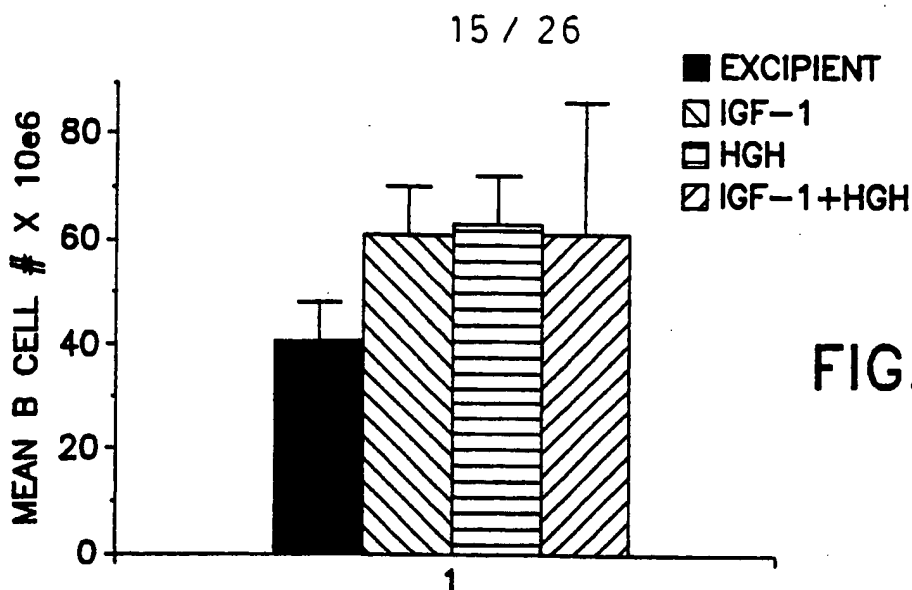


FIG. 17A

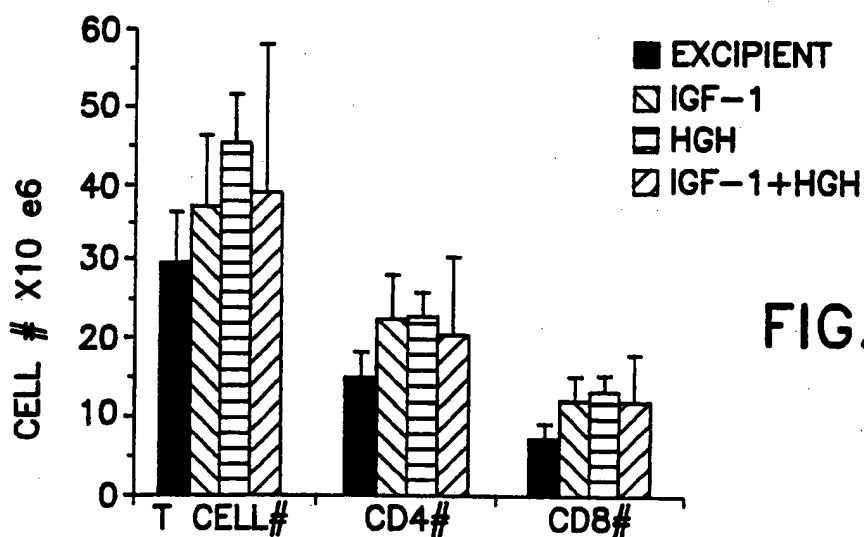


FIG. 17B

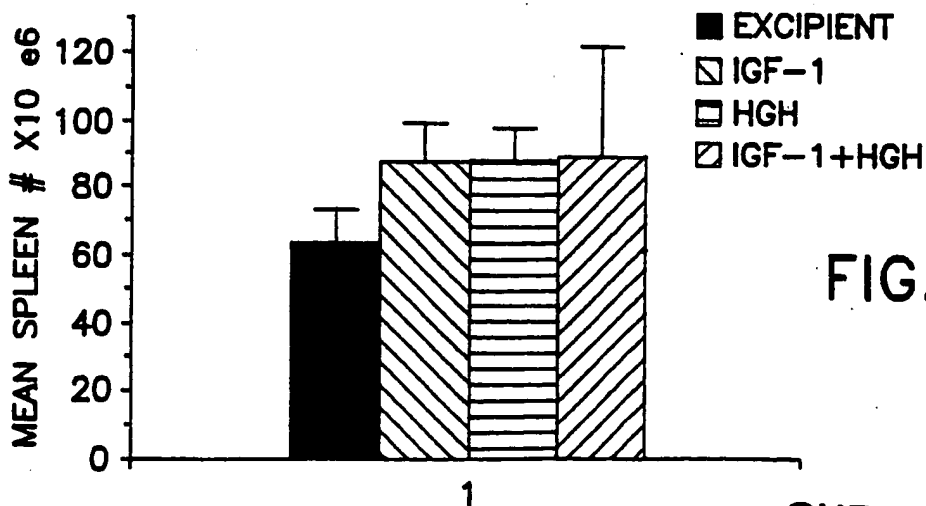


FIG. 17C

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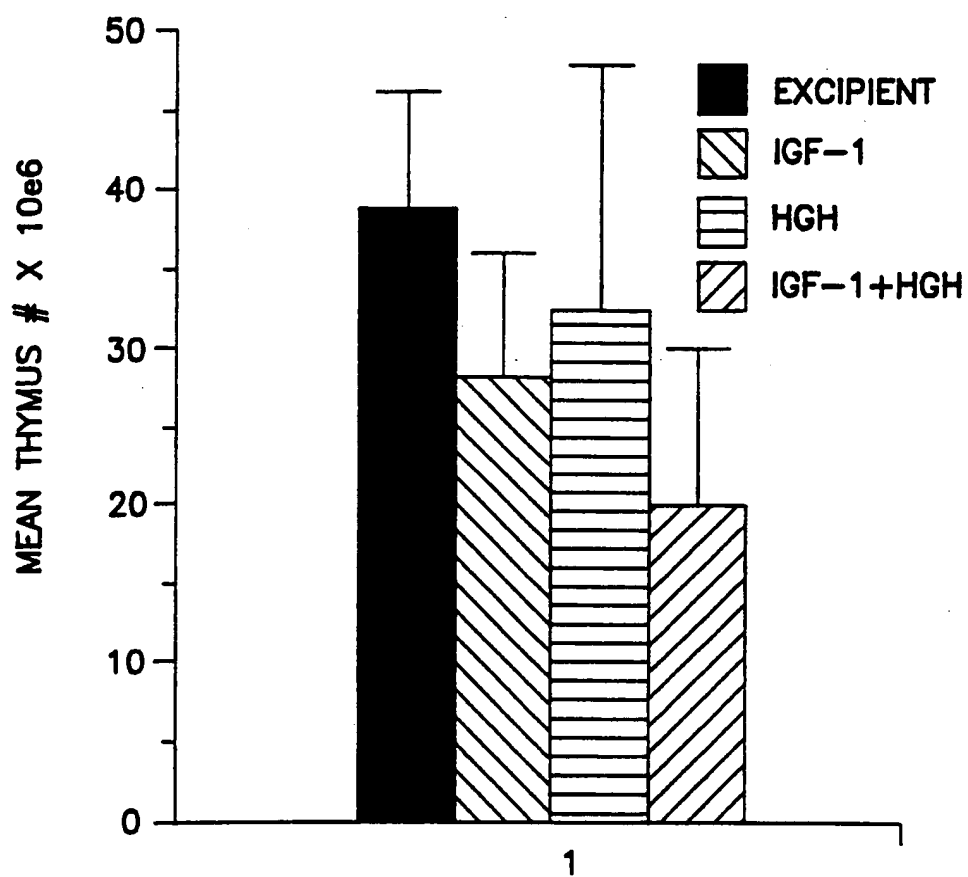


FIG. 18

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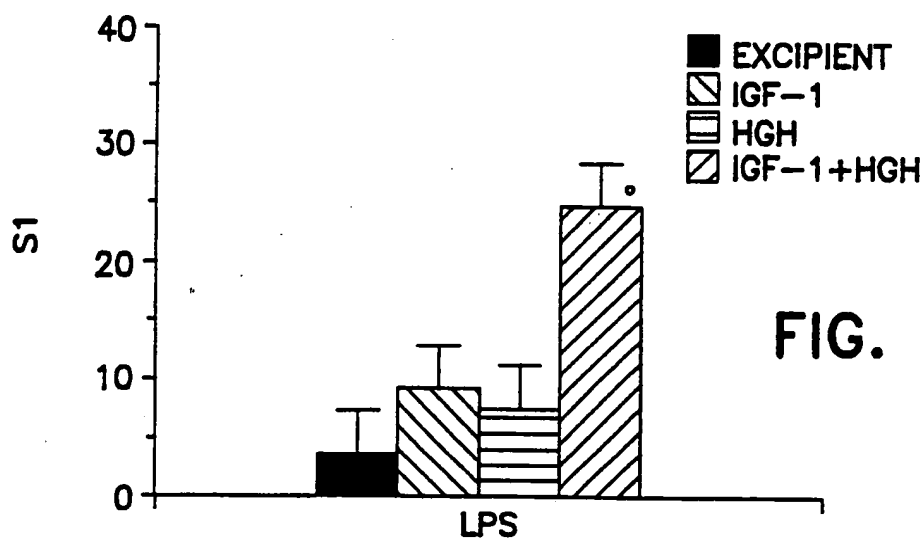


FIG. 19A

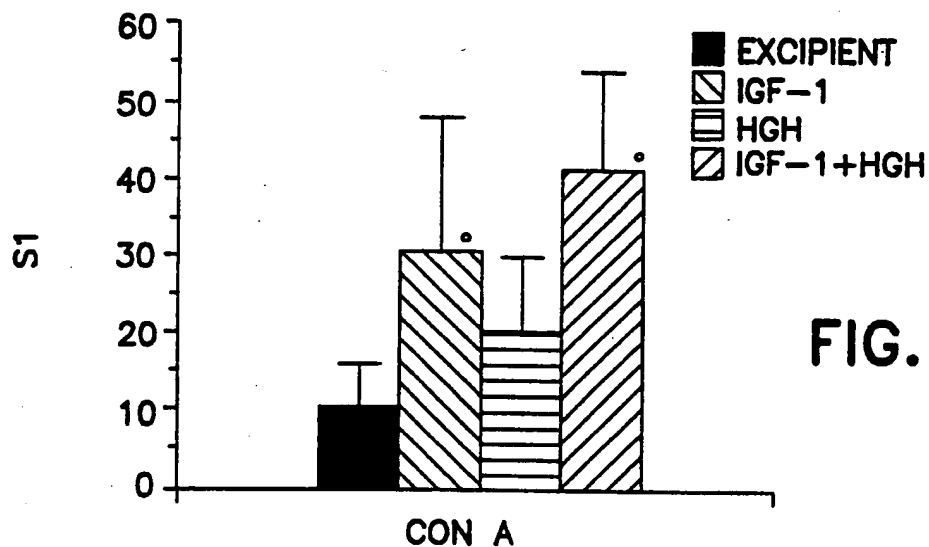


FIG. 19B

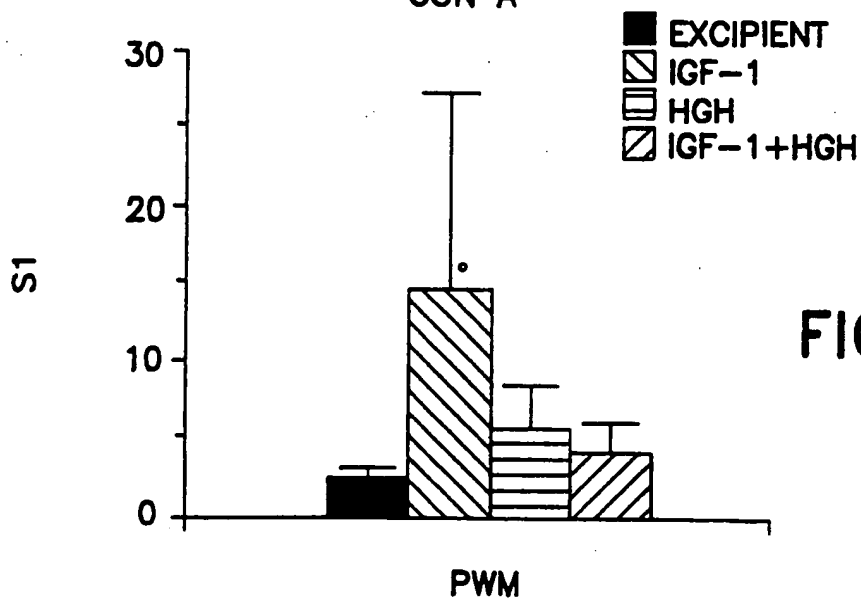


FIG. 19C

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FIG. 20A

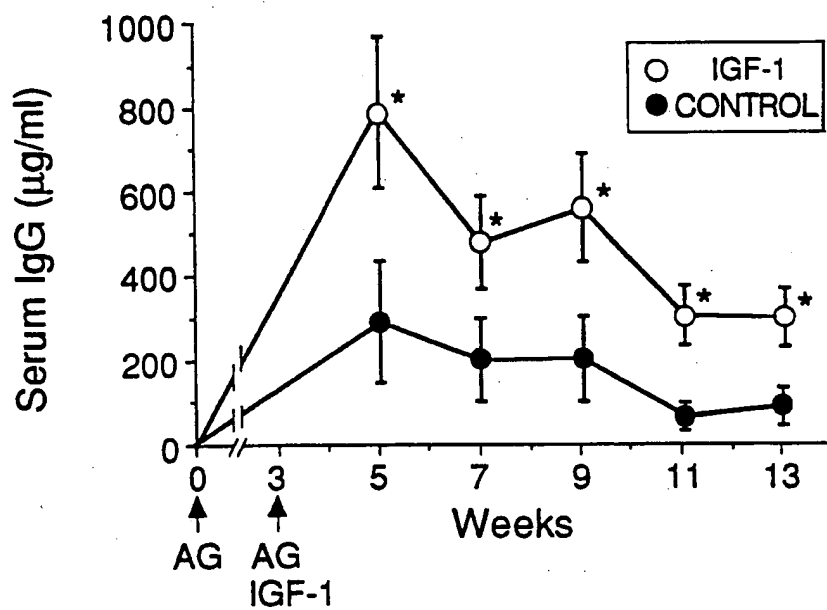
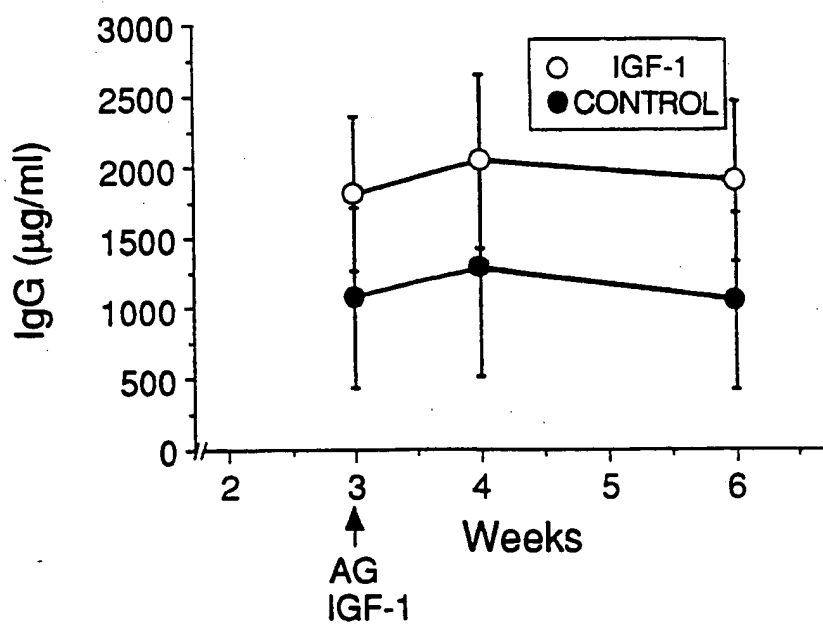
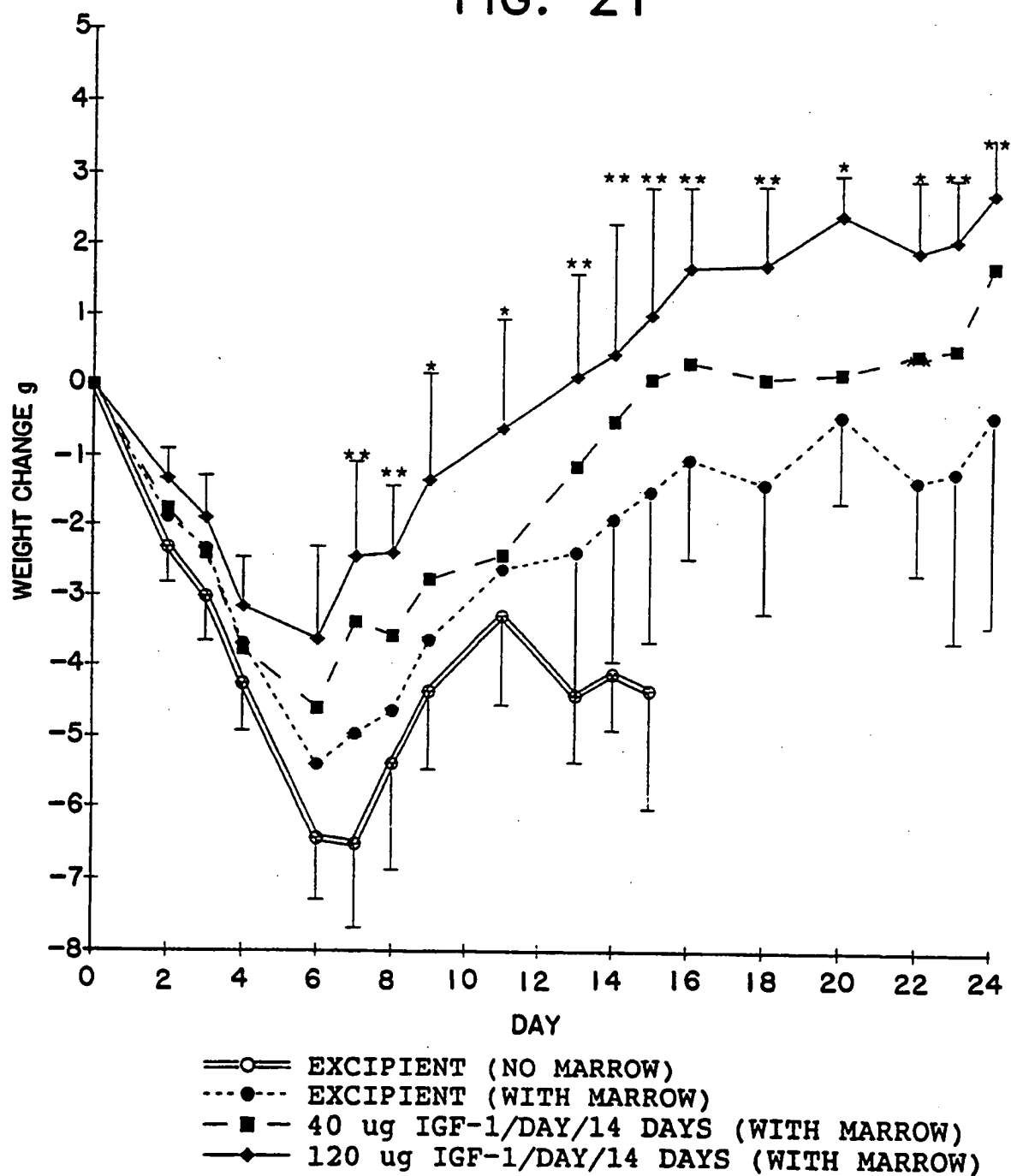


FIG. 20B



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FIG. 21



* SIGNIFICANTLY LARGER THAN ALL GROUPS

** SIGNIFICANTLY LARGER THAN EXCIPIENT GROUPS

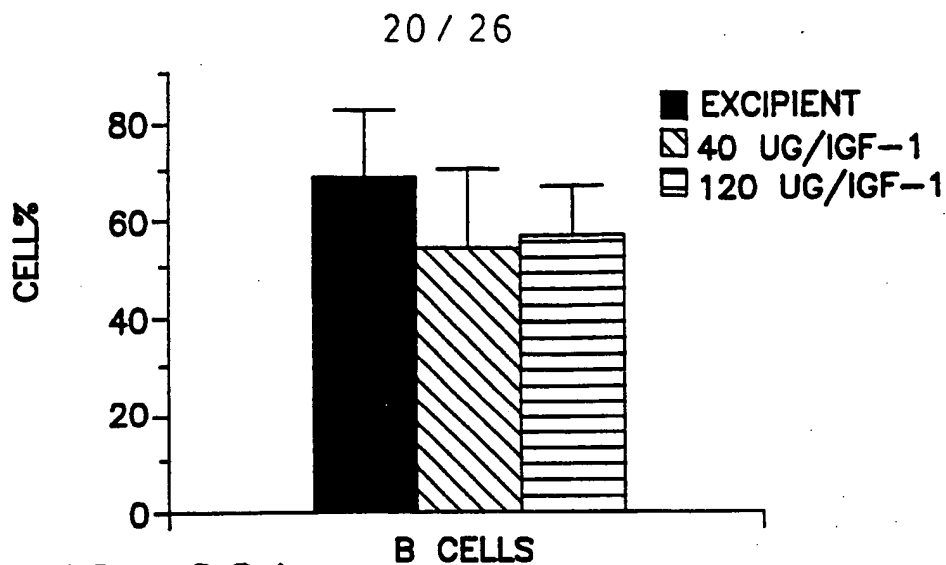


FIG. 22A

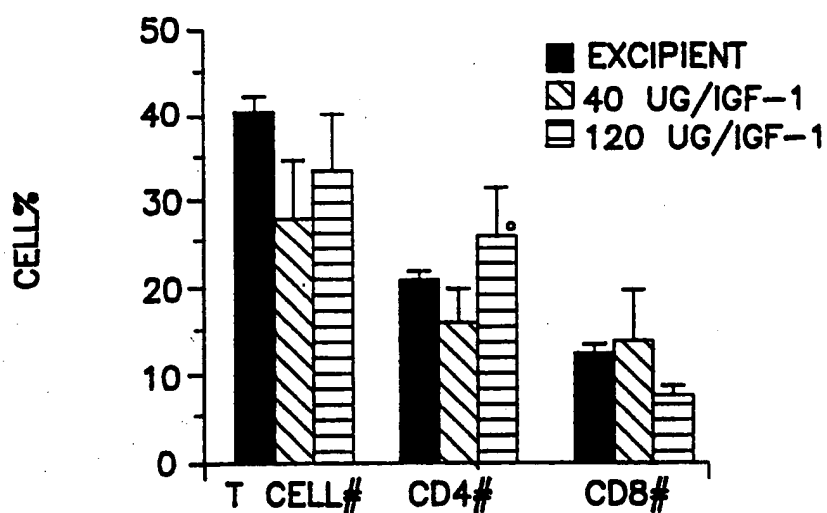


FIG. 22B

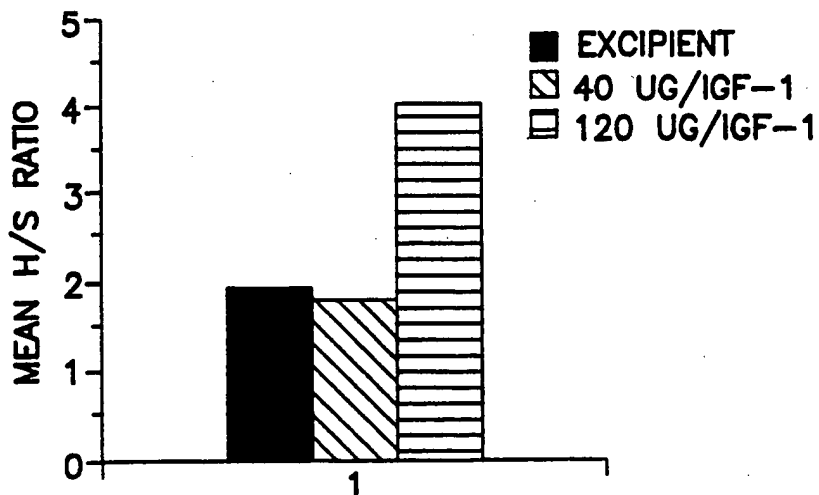


FIG. 22C

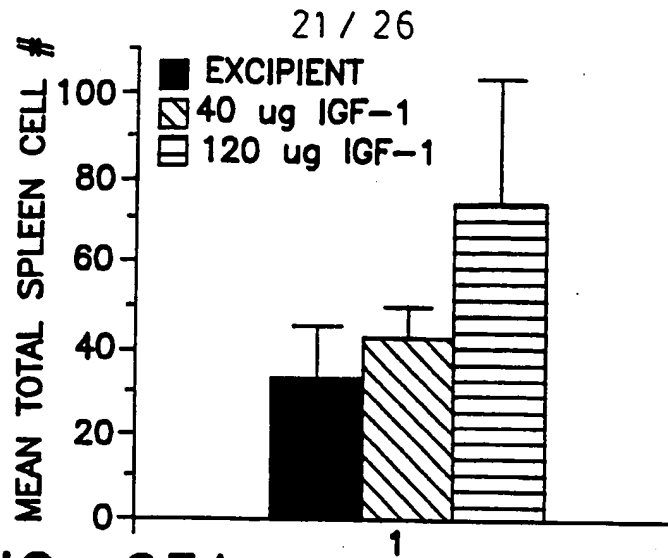


FIG. 23A

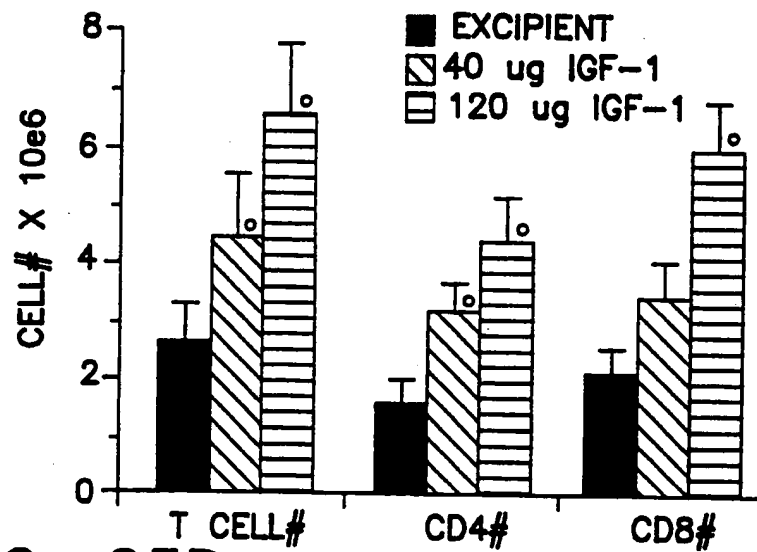


FIG. 23B

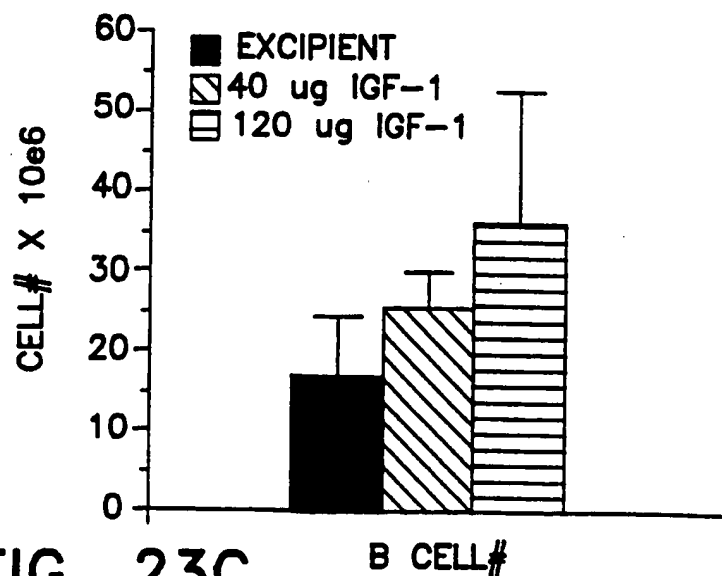


FIG. 23C

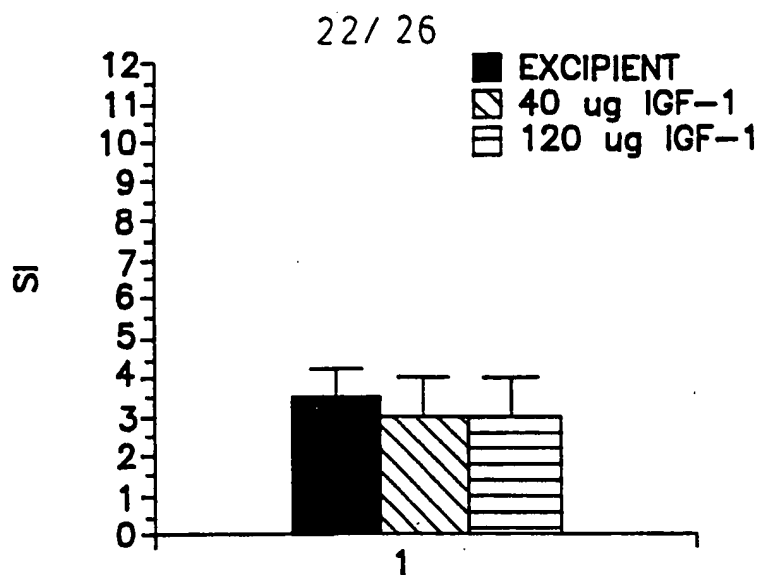


FIG. 24A

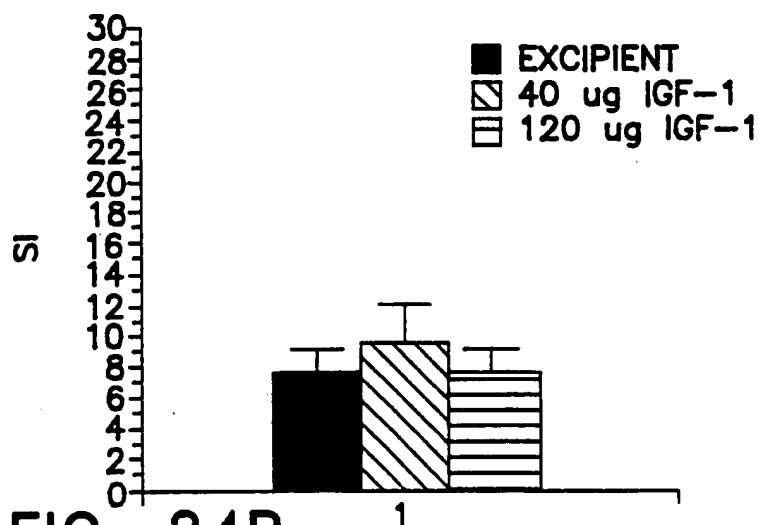


FIG. 24B

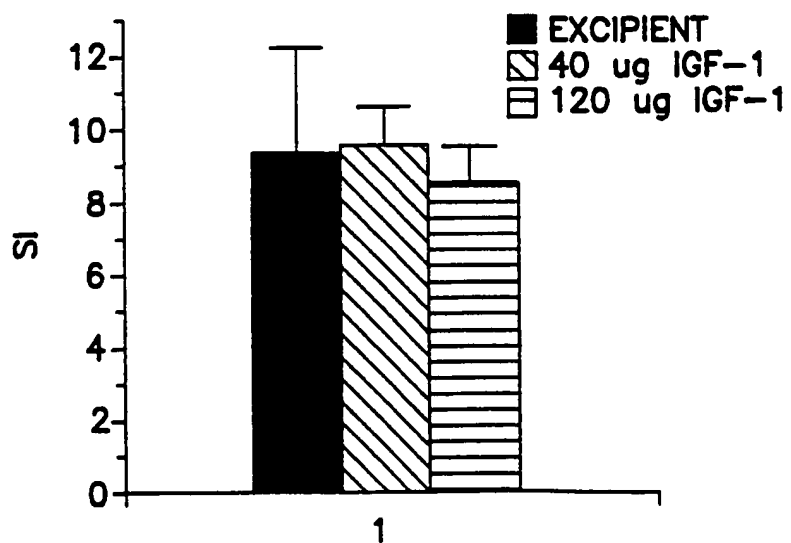


FIG. 24C

SUBSTITUTE SHEET

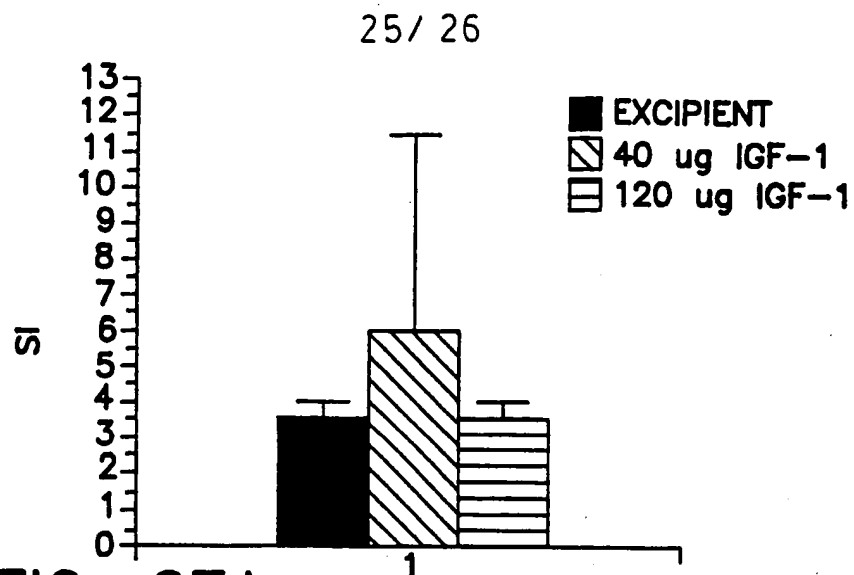


FIG. 27A

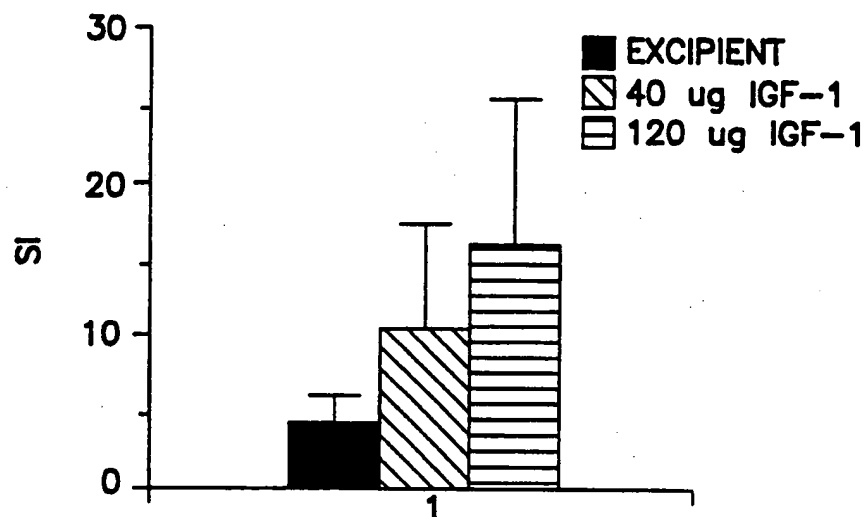


FIG. 27B

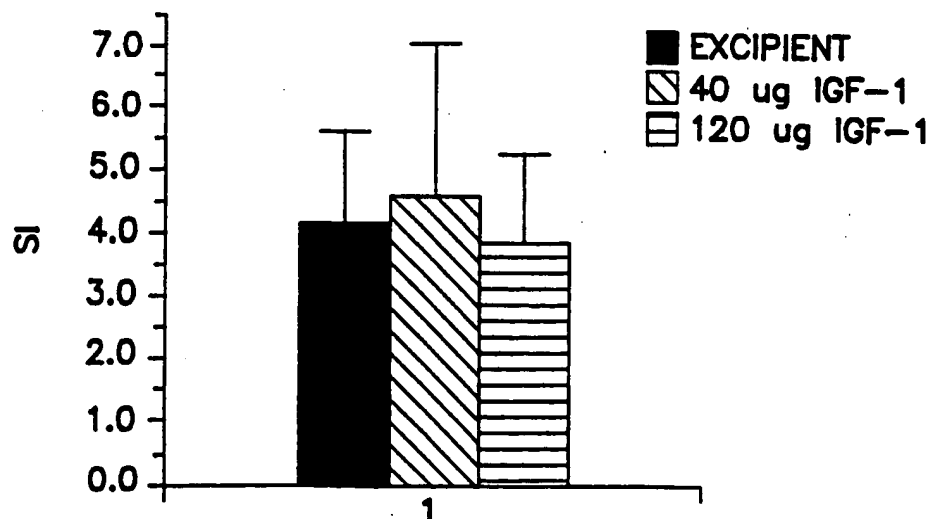


FIG. 27C

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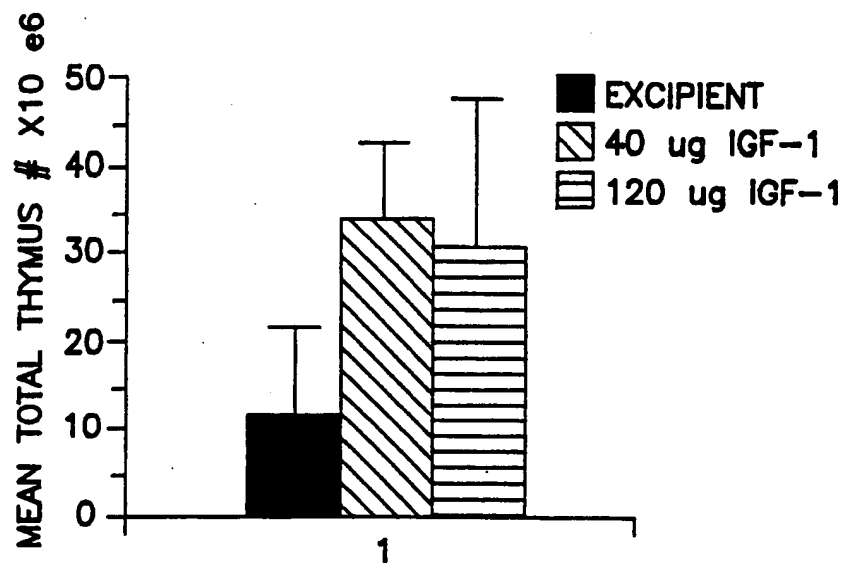


FIG. 28A

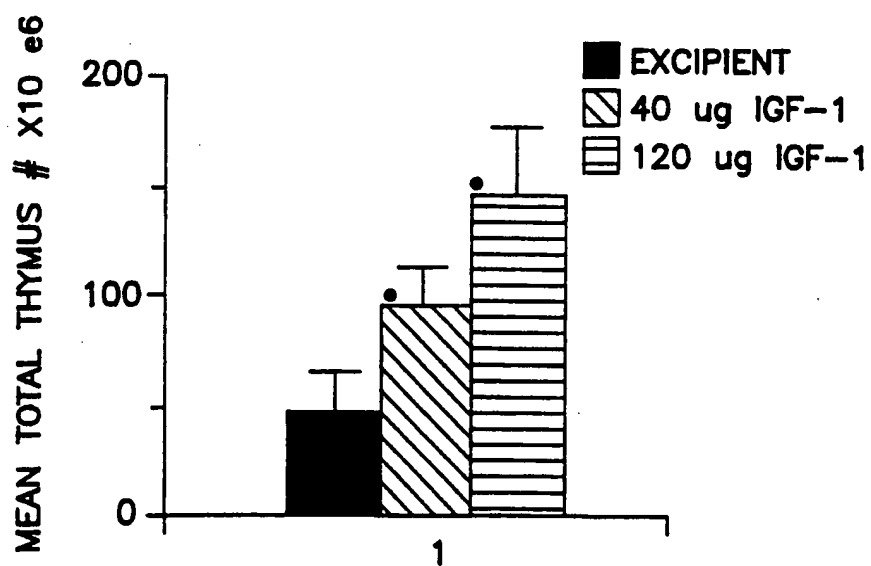


FIG. 28B

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) ⁶		
According to International Patent Classification (IPC) or to both National Classification and IPC		
Int.Cl. 5 A61K37/36; A61K47/34; C07K17/08		
II. FIELDS SEARCHED		
Minimum Documentation Searched ⁷		
Classification System	Classification Symbols	
Int.Cl. 5	A61K ; C07K	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched ⁸		
III. DOCUMENTS CONSIDERED TO BE RELEVANT⁹		
Category ¹⁰	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³
X	EP,A,0 400 472 (SUMITOMO PHARMACEUTICALS COMPANY) 5 December 1990 see page 6, line 51 - line 58 see example 1 see example 49	32
X	WO,A,9 002 559 (PUBLIC HEALTH LABORATORY SERVICE) 22 March 1990 see the whole document	21,23, 25-28
X,P	WO,A,9 116 921 (PUBLIC HEALTH LABORATORY SERVICE BOARD) 14 November 1991 see the whole document	21,23, 25-28
-/--		
<p>¹⁰ Special categories of cited documents:</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</p> <p>"A" document member of the same patent family</p>		
IV. CERTIFICATION		
Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report	
18 SEPTEMBER 1992	4. 10. 92	
International Searching Authority	Signature of Authorized Officer	
EUROPEAN PATENT OFFICE	FERNANDEZ Y BRA F.	

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
X	<p>BICHEMICAL PHARMACOLOGY vol. 38, no. 5, 1 March 1989, NEW YORK pages 705 - 713; KELLEY K.W.: 'Growth hormone, lymphocytes and macrophages' see the whole document</p> <p>---</p>	21,23, 25-28
X,O	<p>THE JOURNAL OF INFECTIOUS DISEASES vol. 164, no. 1, July 1991, CHICAGO pages 188 - 191; STEPHENSON J.R.: 'Adjuvant effect of human growth hormone with an inactivated flavivirus vaccine' Presented in part in the 8th international congress of virology, berlin, september 1990 see the whole document</p> <p>---</p>	21,23, 25-28
A	<p>EP,A,O 308 197 (PITMAN-MOORE INC) 22 March 1989 see the whole document</p> <p>---</p>	21,23-28
A,P	<p>EP,A,O 458 064 (AMERICAN CYANAMID COMPANY) 27 November 1991 see page 2, line 18 - line 24 see the whole document</p> <p>---</p>	32
A	<p>WO,A,8 809 818 (GENENTECH INC) 15 December 1988 cited in the application see example 8</p> <p>---</p>	21, 23-28,32
A	<p>PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA. vol. 85, July 1988, WASHINGTON US pages 4889 - 4893; GULER H.P. ET AL: 'Recombinant insulin-like growth factor I stimulates growth and has distinct effects on organ size in hypophysectomized rats' cited in the application see the whole document</p> <p>---</p>	24

**ANNEX TO THE INTERNATIONAL SEARCH REPORT
ON INTERNATIONAL PATENT APPLICATION NO. US 9204489
SA 60910**

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information. 18/09/92

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A-0400472	05-12-90	CA-A- 2017543	27-11-90
		JP-A- 3095200	19-04-91
		JP-A- 3072469	27-03-91

WO-A-9002559	22-03-90	AU-A- 4324389	02-04-90
		EP-A- 0434749	03-07-91
		JP-T- 4500675	06-02-92

WO-A-9116921	14-11-91	AU-A- 7770991	27-11-91

EP-A-0308197	22-03-89	US-A- 4837202	06-06-89
		AU-A- 2221688	25-05-89
		JP-A- 1287040	17-11-89
		US-A- 5028591	02-07-91

EP-A-0458064	27-11-91	AU-A- 7607591	07-11-91

WO-A-8809818	15-12-88	US-A- 5057417	15-10-91
		AU-B- 616672	07-11-91
		AU-A- 1986588	04-01-89
		EP-A- 0366710	09-05-90
		JP-T- 3500122	17-01-91
